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INTERACTION BETWEEN METHYL MERCURY AND RADIATION EFFECTS ON NERVOUS SYSTEMS

By

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Grant No. R800282

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U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
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The interaction between methyl mercury and ionizing radiation was investigated in a series of experiments using rats, hamsters, and squirrel monkeys to study the effects produced and possible mechanisms of action. Parameters evaluated included several measurements of behavior, brain electrical activity, lethality, blood-brain barrier permeability, neurotransmitter and mercury concentration in various brain areas, and brain histology.

In some cases the effects of the co-insult were less than or at least no greater than at least one of the two insults applied alone.

Possible mechanisms of action include opposite effects of the two insults on the blood-brain barrier, with radiation increasing permeability and methyl mercury decreasing it. Radiation may also elicit a proliferation of peroxisome-like organelles which protect against the effects of methyl mercury.

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This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

Extramural research is an important and necessary supplement to our programs and the fulfillment of our mission. Support for the research objectives detailed in this report resulted from a broadening scientific view of environmental problems; specifically, the need to consider the toxicologic effects of simultaneous exposures to multiple environmental pollutants with common target organs.

John H. Knelson, M.D.
Director,
Health Effects Research Laboratory

ABSTRACT

The interaction between methyl mercury and ionizing radiation was investigated in a series of experiments using rats, hamsters, and squirrel monkeys to study the effects produced and possible mechanisms of action. Parameters evaluated included several measurements of behavior, brain electrical activity, lethality, blood-brain barrier permeability, neurotransmitter and mercury concentration in various brain areas, and brain histology.

In some cases the effects of the co-insult were less than or at least no greater than at least one of the two insults applied alone. Nine kR was less effective than 8 mg methyl mercury per kg of body weight plus 9 kR in producing behavioral decrement in rats 2-4 hours after radiation, producing pyknosis of granule cells of the cerebellum in rats killed 6 hours after radiation, depressing brain norepinephrine levels, and causing death in rats in the first 8 days after radiation. Behavior of female squirrel monkeys was less adversely affected by 300 R plus 6 mg methyl mercury per kg than by methyl mercury alone. Brain electrical activity was similarly affected in rats receiving 4.5 or 9 kR, alone or with methyl mercury. With doses of radiation in the range lethal to 50% of the population in 30 days, the two agents were partially additive.

Possible mechanisms of action include opposite effects of the two insults on the blood-brain barrier, with radiation increasing permeability and methyl mercury decreasing it. Radiation may also elicit a proliferation of peroxisome-like organelles which protect against the effects of methyl mercury.

This report was submitted in fulfillment of Grant Number R800282 by the Texas Woman's University under the partial sponsorship of the Environmental Protection Agency. Work was completed as of February 29, 1976.

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SECTION I

GENERAL CONCLUSIONS

The results of these studies extend our knowledge of effects of methyl mercury intoxication and yield additional information on possible mechanism of action of the agent. However, much remains to be learned in this area, especially with respect to possible differences in response between species.

The results reported herein demonstrate that the interaction between methyl mercury and ionizing radiation is complex. In some cases (several parameters evaluated in rats within 6 hours after the second insult, the squirrel monkey study with a relatively small radiation dose, and the apparent activation of a protective mechanism against methyl mercury-induced damage by radiation) the effect of the co-insult appeared to be less or at least no greater than either insult delivered alone; in many other cases the effects of the two agents were partially additive, but in no case did they appear to be completely additive: i.e., 50% of both insults together did not produce as much damage as 100% of either insult delivered alone.

SECTION II

RECOMMENDATIONS

Possible interactions between agents are potentially important, and the results obtained in this study indicate that more studies involving multiple stresses are necessary in order to properly evaluate the multiple stresses to which human populations are or may be exposed. We recognize that it is not possible to measure all of the parameters which may be affected at various times by all the possible agents, and therefore suggest that the interaction of a limited number of agents currently known to have the greatest potential hazard to man be investigated in detail, with special attention to the nature of the interaction between agents. Hopefully these results will lead to generalizations that could be tested with a limited number of additional agents.

We believe the current data base is inadequate to set meaningful standards for multiple stresses, and that the possible interaction between agents should not be ignored in future standard-setting.

SECTION III

INTRODUCTION

This report presents the results of the major experiments conducted during the 3-1/4 year period supported by the grant. Most of the experiments involved co-insults with methyl mercury and radiation. Some experiments using mercury alone were conducted to provide a basis for the co-insult study; the results of these studies are included in the report at the appropriate locations.

The first series of experiments involved evaluating effects of methyl mercury alone in rats, hamsters and squirrel monkeys. The first major series of experiments involved massive doses of radiation (9kR) and an approximate $LD_{50/30}$ dose of methylmercuric chloride. In this series, the methyl mercury insult preceded radiation by 7 days, and various parameters (including behavior, electroencephalograms, histological analyses, brain mercury concentration and brain neurotransmitter levels) were analyzed in the 6 hour period following irradiation or sham-irradiation. In order to obtain the data required, over 60 rats were assigned to each of 7 treatment groups.

Other studies have involved different temporal relationships between the two insults, varying from methyl mercury preceding radiation by 7 days to methyl mercury following radiation by 4 days. Other studies have been conducted to evaluate effects of lower radiation doses (in the $LD_{50/30}$ range and below).

SECTION IV

METHYL MERCURY EFFECTS IN RAT, HAMSTER, AND SQUIRREL MONKEY: LETHALITY, SYMPTOMS, BRAIN MERCURY AND AMINO ACIDS*

INTRODUCTION

Methyl mercury has become recognized as an exceedingly dangerous by-product of industrial pollution and fungicide application. Neurological disorders involving cerebellar and visual pathway damage have grossly incapacitated humans after ingestion of contaminated shellfish[1] and grain.[2] Similar symptoms have been developed in rats and monkeys,[3] mice, and other test animals.[4] In the resulting voluminous literature,[5] whole body distributions have been clarified in humans and in animal models but the mechanism of damage has remained obscure. Reports of the lethal dose have ranged widely; for example, Peakall and Lovett[6] tabulate values from 30 to 150 mg/kg in rats.

Five points were of primary interest: 1) to describe patterns of lethality produced by single and repeated doses of methylmercuric chloride to rats, hamsters, and squirrel monkeys; 2) to establish a dosage regime which would produce neuro-motor symptoms with an acceptable mortality rate; 3) to attempt to narrow the range of estimated LD50; 4) to determine total mercury in selected brain areas of animals with toxic symptoms; 5) to examine the acumulation of gamma-aminobutyric acid (GABA) and neurotransmitter-like amino acids in the brains of treated animals. The suitability of each species as a model for understanding human damage could then be considered.

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*Manuscript by Betty B. Hoskins and E. W. Hupp which has been submitted to Environmental Research for publication.

MATERIALS AND METHODS

Male Sprague-Dawley rats aged approximately three months and having a weight range of 275 to 320 grams (300 ± 36 g Standard Deviation) were used in most of the experiments. Male Syrian hamsters obtained from F2 and F3 matings for pigmentation inheritance studies had a mean weight of 101.9 ± 14 g. The 15 squirrel monkeys were adult males and females weighing 432 to 1123 grams.

These animals were obtained from and maintained under the standard conditions of the Texas Woman's University animal colony. Two to three monkeys, or five rats, were housed in metal cages. Hamsters were kept in plastic cages with wire tops and corn cob bedding, five to a cage. Rats and hamsters were fed Purina Laboratory Chow with ad libitum water. Monkeys were fed three times a day a diet consisting of Purina Monkey Chow soaked with reconstituted orange juice or milk, fresh fruit, raisins and peanuts.[7] Water was changed twice daily. The animal rooms were maintained at 25°C with a 12 hour light, 12 hour darkness cycle.

Methyl mercury chloride (Alfa Inorganics, Beverly, Mass.) was dissolved in sterile isotonic saline and administered intraperitoneally in one or two ml of saline. Controls received a like amount of isotonic saline. Geometrically spaced doses of 8, 4, 2, or 1 mg per animal were first administered to groups of ten rats and hamsters and to single monkeys. Subsequent series received doses above, below, and intermediate to these increments.

Animals were closely watched for these first 5 hours after injection, then observed daily for morbidity, gait, and appearance changes. They were weighed once a week. Observations were continued for 25 days or until imminent death. At the time of sacrifice, animals were lightly anesthetized with ether and decapitated with a Harvard Apparatus Decapitator (Millis, Mass.). The head was dropped directly into liquid nitrogen. This was necessary since some amino acid levels including GABA, begin to rise within a few minutes after death.[8]

After complete freezing, the entire head was labelled and stored at -29°C in a cold room less than four months. Tissues were fixed in formalin: alcohol: acetic acid and stained with hematoxylin and eosin for histological examination.

The calculation of the LD50 at 24 hours and 30 days (LD50$_{24\ hr}$ and LD50$_{30\ d}$ respectively) was performed by repeated iterations of probit analysis[9] after recalculating individual animal doses to a mg/kg basis and converting % death to probit. These values were checked by use of Weil's[10] tables.

At the time of mercury or amino acid determination, 10 to 100 mg portions of cerebellum, brain stem, or cerebral hemisphere were removed from the skull and weighed. The portions selected were: outer cerebral cortex in the sensory-motor area above the corpus callosum, a central hemisection of cerebellum including vermis and flocculus, and pons and medulla just below the cerebellum. The visual cortex was not included. Matched halves were used, one half for mercury, the other for amino acid analysis.

Total mercury was determined by flameless atomic absorption[11] after overnight digestion with sulfuric acid-permanganate[12] and reduction with a stannous chloride-hydroxylamine solution[13] in a closed system. Duplicate sample readings were compared to standard readings with a Perkins Elmer Atomic Absorption Spectrophotometer set at 2537Å. A standard curve was prepared daily using the same reagents used to treat the samples.

Amino acids were determined by two dimensional thin layer chromatography of dinitrophenol derivatives[14] on ITLC Gelman chromatographic paper. Solvent I was toluene: pyridine: 2-chloroethanol 100:30:60 equilibrated with 60 parts of 0.8 N ammonium hydroxide. A run required approximately 20 minutes; after thorough drying and 90° rotation a run in solvent II (chloroform: benzyl alcohol: glacial acetic acid 70:30:3) required 30 minutes. Spots of GABA, glycine, glutamate, and aspartate were identified by comparison to simultaneously performed chromatograms of commercial DNP-amino acid preparations (Nutritional Biochemical Co.).

Appropriate spots were eluted with 0.010 N $NaHCO_3$ and read on a Perkins-Elmer Spectrophotometer at 360 mU. Samples were automatically transferred from a fraction collector to a flow-through cuvette via a Transferator Programmer (Gilson Medical Electronics). Micromoles of amino acid per gram of brain were calculated.

RESULTS

CALCULATION OF LD 50 $_{24\ hr}$ AND LD 50 $_{30\ d}$

The death rates of rats and hamsters at 24 hours and 30 days after a single intraperitoneal injection of methylmercuric chloride are shown in the sigmoid curves of Figures 1 and 2. (An excessive number of animals was available for this estimation because of the simultaneous collection of tissue for biochemical analysis.) Inconsistency of the percentage lethal response to exponentially spaced doses can be seen and was not eliminated through repeated iterative cycles of probit analysis on rat data. That data showed continued scatter of points around any postulated line (chi square for linearity $.2 > p > .1$). The linearity of the hamster probit curve became valid at $p= .01$. The slope of the curve of response at 24 hours is much steeper for the rat data than for the hamster. That is, the range of doses over which approximately 50% of hamsters die is broader than that for rats. At 30 days, the response of both species was more nearly linear. The LD50's calculated by probit analysis are presented in Table 1 with comparison to the generally agreeing estimates obtained by use of Weil's tables. The LD50 $_{24\ hr}$ was 11.9 mg/kg in the rat and 22.4 mg/kg for both two and three month old hamsters. The LD50 $_{30\ d}$ was 10.1 mg/kg in the rat and 15.2 mg/kg in the hamster.

Nine squirrel monkeys were injected with methymercuric chloride to estimate their lethal dose level (Table 2). Monkeys treated with 2 to 8 mg (3.6 to 17.0 mg/kg) did not die within 24 hours. Thus the LD50 $_{24\ hr}$ is greater than 17 mg/kg. Within one month, a dose of 6.4 mg/kg caused such severe debilitation, tremor, and blindness, as to demand sacrifice; the animal would have starved to death. Heavier animals receiving 4.8 and 5.6 mg/kg survived. Thus the LD50 $_{30\ d}$ can be estimated as between 5.6 and 6.4 mg/kg.

SYMPTOMS OF METHYL MERCURY POISONING AFTER SINGLE AND REPEATED INTRAPERITONEAL DOSES

The response of the three species to a single intraperitoneal treatment in the same dosage range, was strikingly different: Rats exhibited rapid respiratory and vascular symptoms, and did not develop motor damage. Fatally dosed hamsters became comatose but showed no gait abnormalities. Monkeys were not killed within 24 hours by doses (on a mg/kg basis) fatal to 90% of the rodents, and they developed severe neurological symptoms not seen in the rats and hamsters.

Fig. 1. Percentage death rate of rat (●) and hamster (*) 24 hours after a single incremental intraperitoneal injection of methyl mercury chloride.

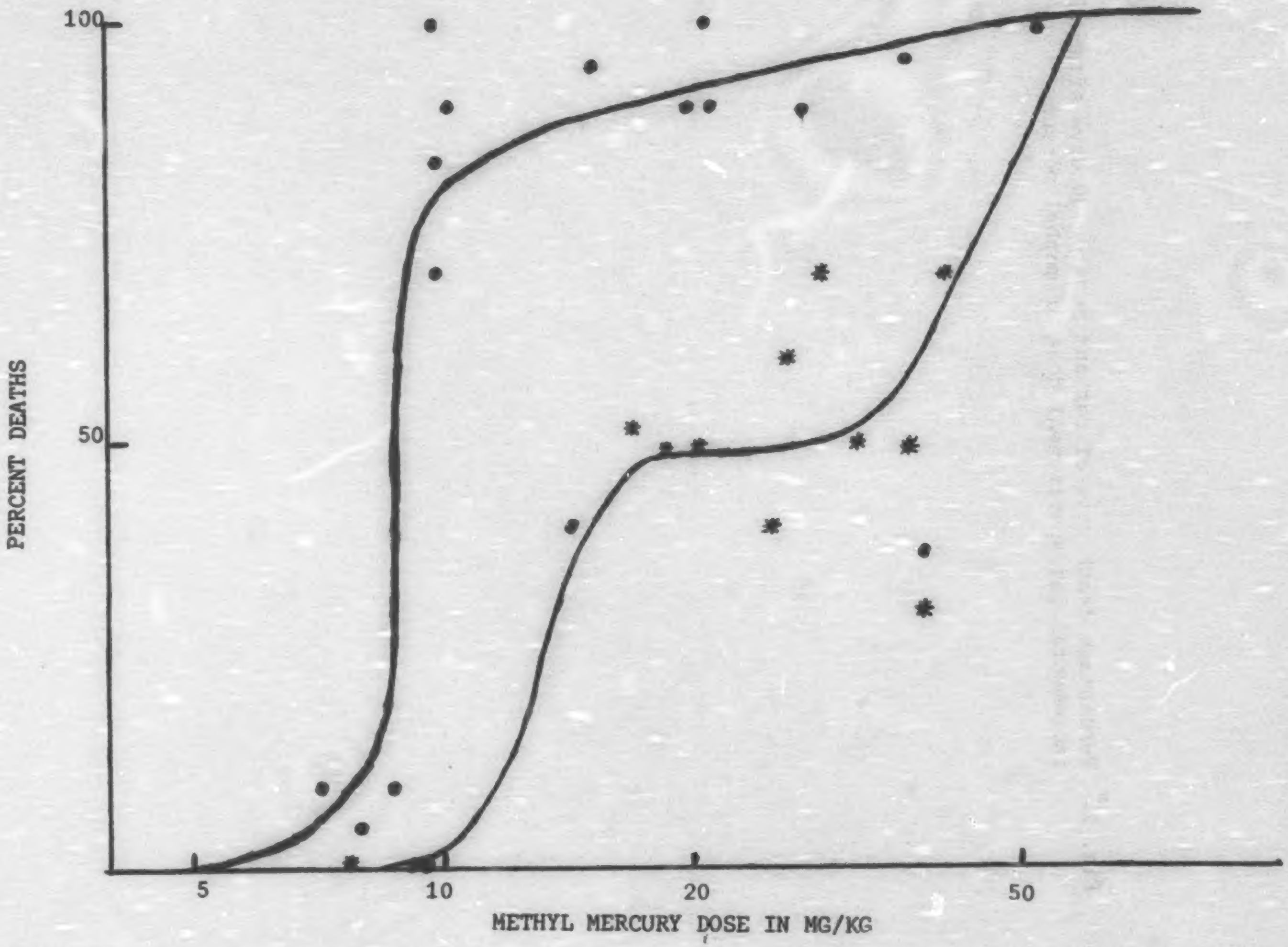
PERCENT DEATHS
100
50
5
10
20
50
METHYL MERCURY DOSE IN MG/KG

Fig. 2. Percentage death rate of rat and hamster 30 days after a single incremental intraperitoneal dose of methyl mercury.

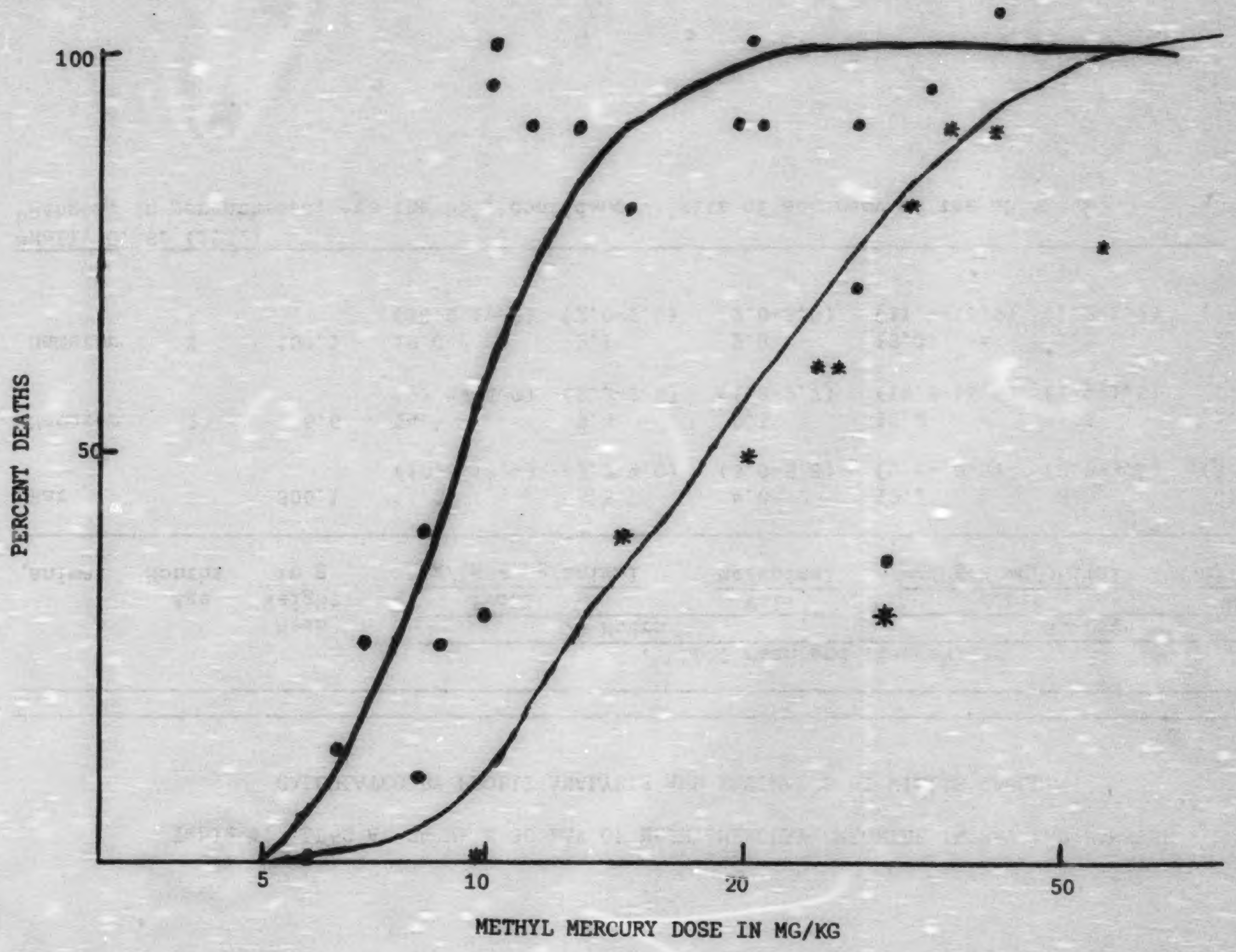
100
50
PERCENT DEATHS
5
10
20
50
METHYL MERCURY DOSE IN MG/KG

Table 1. LD50 AT 24 HR & 30 DAY OF METHYLMERCURY CHLORIDE IN RAT AND HAMSTER CALCULATED BY PROBIT ANALYSIS AND ESTIMATED BY WIEL'S TABLES[a]

Animal	Age Months	Mean Weight in g	LD50 Mean and (Range[b]) at					
			24 Hours			30 Days		
			Probit		Weil	Probit		Weil
			mg/kg	= mg/animal	mg/animal	mg/kg	= mg/animal	mg/animal
Rat	3	300.1	11.9 (10.6-13.4)	= 3.5 (3.2-4.0)	4.0 (3.0-5.3)	10.1 (9.8-10.5)	= 3.0 (2.9-3.1)	4.6 (3.5-6.0)
Hamster	2	96.6	24.5 (23.8-25.0)	= 2.4 (2.3-2.4)	2.5 (1.8-2.7)	15.2 (14.9-15.4)	= 1.5 (1.4-1.5)	1.0 (.74-1.3)
Hamster	3	109.1	19.0 (18.2-19.8)	= 2.1 (2.0-2.2)	2.8 (2.0-3.0)	13.3 (11.4-15.9)	= 1.5 (1.2-1.7)	1.1 (.65-1.8)

[a] Weil, C. S. (1952)

[b] Ranges, in parentheses, are the 95%. confidence limits of accuracy of the LD50

Table 2. INCIDENCE OF MORTALITY, SYMPTOM PRODUCTION, AND BRAIN MERCURY AFTER SINGLE AND MULTIPLE INTRAPERITONEAL INJECTIONS OF METHYLMERCURIC CHLORIDE TO SQUIRREL MONKEYS

Monkey No.	Sex	Body wt (g)	Dose mg/animal	Dose mg/kg	Time of development of severe symptoms[c]	Time of development of moribund condition	Mercury mg/kg Tissue Cer hemis	Mercury mg/kg Tissue Cerebellum	Mercury mg/kg Tissue Brain Stem
Single Dose									
S1	F	471	8.0	16.99	19 hr	54-66 hr	6.76	4.07	3.25
S2	F	423	4.0	9.5	19 hr	66 hr	3.27	3.90	2.08
S3	F	618	4.0	5.6	24 hr	survived[a]	----	----	----
S4	F	467	3.0	6.4	22 hr	25 days	6.15	4.62	3.53
S5	F	620	3.0	4.8	none	survived[a]	----	----	----
S6	F	430	2.0	4.6	transitory[d]	survived[b]	1.86	1.58	0.00
S7	F	533	2.0	3.6	none	survived[a]	----	----	----
S8	F	460	0.0	0.0	none	survived[a]	0.0	0.0	0.0
S9	F	550	0.0	0.0	none	survived[b]	----	----	----
Five Doses									
M1	F	484	5x2	20.7	14 days	15 days	7.03	21.60	13.91
M4	M	743	5x1.5	10.1	14 days	21 days	2.71	4.33	0.62
M5	M	1123	5x1.28	5.7	14 days	21 days	----	-----	-----
M2	F	412	5x1.0	12.4	17 days	21 days	6.50	9.43	11.99
M6	M	915	5x0.86	4.7	5 days	6 days	----	-----	-----
M3	F	543	5x0.75	6.9	21 days	41 days	0.90	1.01	1.24

---

[a] Survived more than 90 days; [b] sacrificed for tissue at 31 days; [c] severe symptoms in monkeys: difficulty in using hand limbs, decomposition of limb movements, intention tremor and overreach; [d] transitory motor symptoms in monkeys: clumsiness of motion between 11 and 25 days after treatment.

In rats, dramatic reaction occurred within 15 minutes after administration of 8, 4, or 3 mg per animal. They became lethargic, heads drooping and eyes dulled. Some of the animals recovered normal color and activity within two to three hours. Others, visibly indistinguishable, progressed to death. The latter showed rapid (60/minute) abdominal breathing, postero-anterior progression of spasticity, and loss of the ability to right and to walk. Two to three hours after injection, respiration became more labored (20/minute), the legs flexed tightly to the body, and the toes developed carpopedal spasm. Symptom progression and the time of death were synchronous for all animals treated with the same dose. Animals treated with less than 3 mg became drowsy but did not expire.

Rats surviving the initial insult showed later general debilitation such as weight loss,[15] abdominal bloating, coarsening and matting of the fur, mucus laden nostrils, and decreased activity; but specific motor changes were not seen. Upon autopsy, the liver appeared pale and encased in tight strands of connective tissue. The intestine was distended with food and gas. Other organs appeared grossly normal.

It was curious to note that all rats which survived the higher doses of 4, 6, or 8 mg/animal and the lowest doses of 0.1 or 0.2 mg per animal recovered from the respiratory distress described and continued to live for one month. Intermediate doses of 2, 2.5, and 3 mg were followed by further deaths throughout the month. The time until death increased, and the percentage of animals dying decreased, with decreasing dose.

Single doses of 0.2 to 8.0 mg of methylmercuric chloride to hamsters simply accentuated their normal cyclic chattering, and quarreling, and sleeping. Just after injection, the hamsters appeared drowsy; only a mild cyanosis distinguished treated animals from the normal nap behavior of controls dosed with saline. Most responded to poking with biting and fussing. Deep cyanosis was not seen; a comatose condition progressed to death about three hours after injection. Survivors showed a gradual and steady weight loss. A few deaths occurred in each dosage level during the following 30 day interval. No gait problems developed.

No monkeys died within the first 24 hours after a single treatment, although the dosage levels on a mg/kg basis were comparable to those killing 80 to 90 percent of the rats and 50% of the hamsters. (Table 2) By 19 hours, animals dosed with 4 to 8 mg per animal (5.6 to 17 mg/kg) became huddled and indifferent to prodding. Death ensued by 66 hours. An animal dosed with 3 mg (6.4 mg/kg) became lethargic, clumsy, and uncoordinated at 22 days, and deteriorated rapidly. By 24 days, she became blind, drew her forelimbs jerkily to the mouth, and remained rigid when handled. Sacrifice was essential. Treatment of 3 animals with 2 or 3 mg (3.6 to 4.8 mg/kg) was followed by transient lethargy, minor motor difficulty, and recovery to normal within 26 days.

Four and five doses of methyl mercury were administered to series of rats, hamsters, and monkeys to establish a dosage which would produce symptoms within an acceptable mortality rate. In Table 3 the results of rat series shows that a regimen of at least 5 daily doses of 2 mg each (approximately 34 mg/kg) were required to cause the beginning of motor symptoms (previously described as partial tucking of the hindlegs when lifted by the tail) 12 to 15 days after the initiation of injections. Definite signs (hindlimb tucking, paw flexion, and walking high on the legs) were seen within three weeks after the beginning of injections in about 40% of the survivors at the time (10-20% of the original sample). An additional 27% of survivors exhibited moderate symptoms; the remaining 33% had a normal gait. All were bloated, diarrhetic, and matted. Four doses, or five doses of 1 mg produced no motor symptoms in an adult or junevile (series C) rats.

Five doses of 1 mg each to hamsters produced a high mortality rate (76%) and a low symptom rate (4%), parallelling the high death rate after a single 5 mg dose (Fig 2). Consequently multiple two mg doses were not administered.

The effects of repeated doses on 3 female and 3 male squirrel monkeys are shown in Table 2. All cumulative doses from 4.7 to 20.7 mg/animal were followed by the same degenerative pattern. The earliest appearance of symptoms (toes curled, muscle spasms of the limbs, anteroposterior rocking) occurred 5 days after injection, in a large male. All other developed symptoms at 14 - 21 days. Deterioration of coordination and vision was rapid.

MERCURY LEVELS IN BRAINS OF RATS AND MONKEYS

Total mercury was determined in less than 100 mg portions of sensory-motor cortex, cerebellum, and pons and medulla of individual rats sacrificed 21 days after treatment with methylmercuric chloride. The levels are presented in Table 4 as mean values for groups of three rats treated with the same dose. A range of mercury accumulation is indicated by those means and standard deviations. A significant (at 95% confidence limits) difference was seen between levels in animals with and without symptoms and between both those groups and the controls. Individual rats with motor impairment contained 8.8 to 17.5 µg/g cerebral hemisphere, 3.7 to 18.6 µg/g cerebellum, and 4.1 to 17.5 µg/g brain stem. There was not a significant difference (95% CL) in accumulation between those three areas. Animals with only nonspecific symptoms of bloating and diarrhea contained 2 to 15 µg/g cerebrum, 6 to 17 µg/g cerebellum and 2 to 2.7 µg/g brain stem. After a single dose, the highest mercury levels detected were less than those after multiple injections. Mercury was detected in the brains of controls housed in the same room with injected animals, at levels of 0.2 to 0.8 µg/g cerebral hemisphere, and 0.0 to 0.7 µg/g brain stem.

Table 3. INCIDENCE OF MORTALITY AND SYMPTOM PRODUCTION IN RATS AFTER MULTIPLE INJECTIONS OF METHYLMERCURY

mg/animal	Series No.	No. Animals	Mean Weight g	% All Animals Dead after Doses 1	2	3	4	5	% All animals dead in 21 days	% All Animals with symptoms at 21 days
2 mg weekly for 6 weeks	A	10	419.5	40	50	50	60	50	50	0
2 mg daily for 5 days	D	10	289.0	0	0	0	20	20	40	20
	E	10	376.7	0	0	20	50	50	60	10
	F	74	293.2	0	0	3	5	11	57	13
	G	50	317.8	18	40	48	52	64	76	14
1 mg daily for 5 days	D	10	298.3	0	0	0	0	0	0	0
	E	10	381.7	0	0	0	0	10	10	0
	C	10	122.6	0	0	20	50	70	80	0
1.5 mg daily for 4 days	B	50	295.6	0	0	0	0	0	8	6

Table 4. MEAN TOTAL MERCURY CONCENTRATIONS PER GRAM OF BRAIN IN THREE AREAS OF RAT BRAIN AFTER SINGLE AND MULTIPLE INJECTIONS OF METHYLMERCURY

Dose		No. animals	Motor symptoms	Brain Area		
mg/animal	mg/kg			Cer. Hemis. µg/g	Cerebellum µg/g	Brain Stem µg/g
Five Doses of 2 mg Each						
10.0	34.0	3	slight[b]	13.74 ± 4.40[a]	6.49 ± 4.07	7.47 ± 4.20
10.0	34.0	3	yes[c]	10.18 ± 2.14	10.28 ± 3.00	8.06 ± 2.94
10.0	34.0	3	beginning[d]	11.21 ± 5.00	16.97 ± 5.65	11.01 ± 5.67
10.0	34.0	3	non-specific[c]	6.88 ± 6.82	12.81 ± 5.76	2.36 ± 0.40
0.0	0.0	3	no	0.18 ± 0.32	0.00	0.00
Single Dose						
4.0	13.3	3	no	3.83 ± 4.55[a]	1.85 ± 2.37	1.12 ± 1.40
2.5	7.8	3	no	2.22 ± 1.24	1.67 ± 0.93	1.15 ± 0.42
1.0	3.3	3	no	0.48 ± 0.30	0.41 ± 0.65	0.45 ± 0.74
0.0	0.0	3	no	0.47 ± 0.30	0.22 ± 0.38	0.25 ± 0.43

[a] Mean ± standard deviation

[b] Slight motor symptoms in rats: partial tucking motions of hindlimbs when lifted by tail

[c] Definite motor symptoms in rats: hindleg tucking when lifted by tail; paw flexion

[d] Beginning symptoms: loss of weight

[e] Nonspecific symptoms: bloating, diarrhea

Hamster mercury levels were not determined since symptom production was at such a low level. The levels of mercury in the brains of single monkeys which had received single and multiple doses of methylmercury are summarized in Table 2. There was little difference in level of accumulation between brain areas at any dosage range except the highest, 5 doses of 2 mg each (20.7 mg/kg). Mercury was accumulated in all three areas of the brain at the same levels after a single dosing. No mercury could be detected (less than .001 ppm) in the brain tissue of a control monkey housed in the same room with treated animals.

AMINO ACID LEVELS IN BRAIN TISSUES OF RATS AND MONKEYS

Levels of selected amino acids suspected of neurotransmitter function were determined in 10 to 100 mg portions of the cerebral hemispheres, cerebellum, and brain stem of rats and monkeys opposite to the areas used for mercury determination. When 20 to 30 µl aliquotes of DNP-derivative/extract were used as the total spot volume, amino acids present in concentrations less than 1 µM/g of tissue were not consistently visualized. The amino acids present in greater concentration, especially glutamate, aspartate, glycine, and GABA, were easily visible and discrete. Such spots were eluted and quantified by spectrophotometric comparison to DNP-amino acid standards. A possible artifact, DNP-hydroxide which may be present when heptane bromobenzene washing is incomplete, was rigorously avoided by washing and by excluding all spots with a value greater than cf 0.55 in Solvent I. (GABA Rf values were 0.44 in Solvent I and 0.77 in Solvent II; DNP hydroxide has an Rf of 0.60 in Solvent I and 0.85 in Solvent II according to Shank and Aprison,1960).

In rats (Table 5) no marked differences were found in glutamate, glycine, aspartate, or GABA levels of control and symptomatic rats and in the three brain areas. In the monkeys, however, (Table 6), increased GABA levels and marked reductions of glutamate, aspartate and glycine levels were seen in all dosed brains. Glycine became so low as to be indectectable. An exception was found in a monkey dosed with the lowest single dose (6.42 mg/kg); aspartate levels were normal in cerebellum. Levels of those amino acids in control monkeys were in comparable ranges with previously published ranges.[16] GABA levels showed a moderate rise in cerebral hemispheres of multiply dosed animals; in the cerebellum and brain stem, levels were 2 to 100 times the control. Singly dosed animals displayed only moderate rises in GABA content in the hemisphere and inconsistent cerebellar and brain stem levels.

DISCUSSION

In our study, a range of lethal doses and some non-linearity of the regression curve continued even though large numbers of animals were

Table 5. MEAN AMINO ACID LEVELS PER GRAM OF BRAIN IN THREE AREAS OF RAT BRAIN AFTER TREATMENT WITH FIVE DOSES OF TWO MG OF METHYLMERCURY

Amino Acid	Dose mg/animal	Dose mg/kg	Series	No. An.	Motor Symptoms	Brain Area: Cer. Hemis. µM/g	Brain Area: Cerebellum µM/g	Brain Area: Brain Stem µM/g
Glutamate	10.0	34.0	F	3	yes[b]	3.23±0.74[b]	3.27±0.25	2.64±0.37
	10.0	34.0	G	3	yes	3.25±1.30	0.67±0.55	0.52±0.09
	10.0	34.0	G	3	Slight[c]	1.72±0.30	3.93±0.63	5.35±0.90
	0.0	0.0	F	3	no	13.41±1.55	3.70±1.50	1.10±0.73
Aspartate	10.04	34.0	F	3	yes	1.77±0.14	6.07±0.63	2.34±1.60
	10.0	34.0	G	3	yes	1.72±1.10	1.03±0.72	0.29±0.04
	10.0	34.0	G	3	slight	3.50±0.04	2.47±0.30	0.76±0.30
	0.0	0.0	F	3	no	2.20±0.22	3.70±2.50	2.17±0.29
Glycine	10.0	34.0	F	3	yes	nd[d]	0.17±0.07	nd
	10.0	34.04	G	3	yes	3.36±2.80	0.56±0.55	0.50±0.60
	10.0	34.0	G	3	slight	1.11±1.60	1.88±0.57	1.10±0.11
	0.0	0.0	F	3	no	1.49±0.40	0.33±0.22	0.33±0.10
Gamma-Amino Butyrate	10.0	34.0	F	3	yes	5.00±5.21	1.64±0.75	3.94±0.30
	10.0	34.0	G	3	yes	0.32±0.31	4.22±4.60	3.09±1.24
	10.0	34.0	G	3	slight	8.47±1.45	4.41±1.21	1.24±0.19
	0.0	34.0	F	3	no	4.56±3.10	3.84±4.30	3.25±1.37

Table 6. AMINO ACID LEVELS PER GRAM OF BRAIN IN THREE AREAS OF SINGLE MONKEY BRAIN AFTER TREATMENT WITH SINGLE AND MULTIPLE DOSES OF METHYLMERCURY

Dose		Motor	Brain Area		
mg/animal	mg/kg	Symptoms	Cer. Hemis. µM/g	Cerebellum µM/g	Brain Stem µM/g
Glutamate					
8.0	16.99	no[a]	1.46	nd[b]	0.09
4.0	9.46	no[c]	0.33	1.16	nd
3.0	6.42	yes[d]	0.40	nd	nd
5x2.0	20.66	yes	nd	0.65	nd
5x1.5	10.09	yes	0.24	0.49	0.03
5x1.0	12.14	yes	nd	0.39	nd
5x0.75	6.91	yes	0.73	0.40	0.56
0.0	0.0	no	1.54	3.95	4.56
Aspartate					
8.0	16.99	no, died	0.20	nd	0.003
4.0	9.46	no, died	0.16	0.20	nd
3.0	6.42	yes	0.16	1.52	nd
5x2.0	20.66	yes	nd	nd	nd
5x1.5	10.09	yes	0.59	nd	0.003
5x1.0	12.14	yes	nd	nd	nd
5x0.75	6.91	yes	0.41	nd	1.80
0.0	0.0	no	2.70	1.42	1.70
Glycine					
8.0	16.99	no,died	0.20	nd	nd
4.0	9.46	no,died	0.40	nd	0.002
3.0	6.42	yes	1.05	nd	nd
5x2.0	20.66	yes	nd	nd	nd
5x1.5	10.09	yes	0.54	nd	0.011
5x1.0	12.14	yes	nd	nd	nd
5x0.75	6.91	yes	0.66	0.014	0.32
0.0	0.0	no	0.44	0.94	0.40
Gamma-Amino Butyric Acid					
8.0	16.99	no,died	4.13	3.0	0.84
4.0	9.46	no,died	3.22	9.2	8.90
3.0	6.42	yes	1.18	---	9.00
5x2.0	20.66	yes	3.0	10.47	4.88
5x1.5	10.09	yes	4.6	14.07	22.60
5x1.0	12.14	yes	---	21.48	4.76
5x0.75	6.91	yes	2.01	3.60	15.53
0.0	0.0	no	1.00	5.47	0.33

[a] Died by 19 hrs after dosing. [b] nd=not detectable. [c] Died by 19 hrs after dosing. [d] Definite motor symptoms in monkeys: difficulty in using hind-limbs, decomposition of limb motion, intention tremor and overreach.

used over a six month period. The hamster death rate showed a plateau over a range of doses. Previous reports have ranged widely; Swensson and Ulfvarson[17] tabulated oral methylmercury dicyanimide as having a reported LD50 of 26 and of 32 mg/kg. Ulfavarson[18] lists 400 mg of methylmercury hydroxide as a "1 percent LD50 dose", and Swensson[19] reported a lethal intraperitoneal dose of 10 mg/kg of methylmercuric chloride dissolved in oil. Peakall and Lovett[20] summarized available figures as indicating an LD50 of 30 to 150 mg/kg in rats. An LD50 for hamsters and monkeys has not previously been stated.

Given the continuing inconsistency of results, not all of which can be attributed to omission of information on time interval, route of administration, anion, age, and sex of the animal, one must question the method of experimental procedure and method of LD50 estimation. Finney[21] cites studies showing all current LD50 estimation methods to produce similar estimates over a middle range of doses. It may be that the dose response pattern to methyl mercury is in fact more complex than a simple exponentially-linear nonthreshold relationship. More than one death mechanism may be involved, summating at intermediate doses. Clearly death within 24 hours was related to respiratory depression. Later, nonspecific body thinning and bloating probably involved cumulative inhibition of liver sulfhydryl-containing enzymes[22] and brain cofactors.[23] But such inhibition cannot account for the description of brain tissue deep in sulci as anemic or for the greater effect on hindlimb innervation[24] and activity. Further, the dose response may not be normally distributed; methyl mercury poisoning may be a threshold phenomenon in which there are doses below which no animal is killed with the designated time period. It may also be that rats, even from a long established colony and strain, are not evolutionarily homogenous with respect to methyl mercury sensitivity, and susceptibility and uptake may vary as it apparently does in humans.[25]

The pattern of damage to humans and other mammals is becoming well defined and amenable to behavioral analysis.[26] The initial study of Hunter et al.[27] described both classic human and animal symptoms. Slurred speech, numbed and tingling fingers, and limb weakness progressing to ataxia, concentric narrowing of the visual fields, fine motor disability, and memory difficulties were seen in the first cases. Similar degeneration has been described after ingestion with food[28] and after respiratory exposure of laboratory workers[29] and seed dusters.[30] Volunteers[31] and high fish diet consumers have reported nonspecific irritability and EEG changes. In animals, inability of the rat to hold the head erect[32] and increased critical fusion intensity in monkeys[33] have been established as early signs. Hunter et al.[34] described the progression in rats and monkeys; clumsiness of the reddened cold hindlegs and weight loss were the first discernable changes. As the animal became moribund, the forelegs became involved. Irritability, ataxic changes, and leg cross when lifted by the tail followed sub-lethal doses.[35] The monkey shows degenerative patterns similar to humans.[36] Surprisingly, the respiratory distress and liver changes have not been previously commented upon.

Neurological damage was produced in rats only by multiple doses; monkeys responded to single doses of greater than 6 mg. Most previous work on rodents is in agreement.[37] Only Yoshino et al.[38] obtained damage in immature rats with a massive 7.5 mg/100 g body weight. It is emerging that the immature animal shows a different pattern of damage than the adult.[39] Our pilot study too showed juvenile rats less likely to develop gait problems than were adults. The percentage of adult rats displaying symptoms varied from series to series, as previously reported by Swensson and Ulfvarson.[40] Yet Klein et al.[41] found 7 doses of 10 mg/kg to consistently produce symptoms without lethality at 14 days.

The effect of distributing the dose over a five day period varied with the species. In rats, similar lethality was produced by 10-12 mg/kg as a single dose and by 33 mg/kg delivered as 5 portions. Perhaps detoxification mechanisms, especially via the kidney, are immediately effective. In hamsters, the susceptibility to cumulative doses administered daily for five days was nearly as great as that to the dose given as a single injection, but gait problems were rare. A squirrel monkey was killed by 5.7 mg/kg given as five doses while another survived a single 5.6 mg/kg. After humans ingested an estimated 3.9 mg per day for 100 days,[42] juveniles 8-13 years old remained severely handicapped; a 20 year old recovered some speech and mobility. Adult members of the family remained asymptomatic. The monkey then would seem a useful if more sensitive model for the human than is the rodent.

In the three species studied, the $LD50_{30\ \text{days}}$ decreased with increase in body weight: hamster 15 mg/kg, rat 10 mg/kg and monkey approximately 6 mg/kg. Within a species, the larger animals appeared more sensitive than predicted on a mg/kg basis.

It is always necessary to demonstrate that mercury has in fact crossed the blood-brain barrier and is present in animals presenting neurological symptoms attributed to its presence. In this study, mercury was demonstrated in samples of less than 100 mg of single brain areas of single rats and monkeys. It would be feasible to dissect free nuclear areas in the monkey brain. In both rats and monkeys, mercury was present in cerebral hemispheres, and in the brain stem. Variation in content between animals was significant, but variation between areas was not. The results of the present experiments indicating symptomatic animals have greater than 8 μg Hg/g brain agree with previous estimates of threshold mercury levels. Berglund and Berlin[43] reported 8 μg/g tissue is necessary to produce symptoms; Evans et al.[44] quote Suzuki and Miyama as finding 10 ppm to cause head lag in mice, and Berlin et al.[45] report 9 μg/g tissue as the calculated level producing an effect in monkeys.

Real differences in levels of neurotransmitter-like amino acids and of the inhibitory neurotransmitter GABA, were seen between control and treated monkeys. But levels were not significantly different in rats.

Similarly, Spyker[46] reported no change in choline acetyl-transferase and choline esterase in rats. Detected levels were consistently higher for GABA, and lower for glycine, than those in the literature, despite immediate freezing of heads. Glutamate and aspartate levels were consistent with the previous literature. It is curious that the GABA level of methylmercury-treated animals were *raised* above those of concomitant controls. Mercurials cause decreased synthesis *in vitro* of GABA,[47] presumably by inhibiting the action of glutamate decarboxylase and stimulating the degradation of GABA by transamination with alpha-ketoglutarate.[48] Shank and Aprison[49] however, believe glycine and GABA to exist in at least two metabolic pools, one more active than the other. Further analysis of defined portions of monkey brains, correlated with behavioral changes, should be of interest.

SUMMARY

The $LD50_{24\ hr}$ of methylmercuric chloride was estimated as 11.9 mg/kg in the rat, 22.4 mg/kg in the hamster, and greater than 17 mg/kg in the squirrel monkey. At 30 days, the LD50 was 10.1 mg/kg in the rat, 15.2 mg/kg in the hamster, and estimated as 4.7 to 6.4 mg/kg in the monkey. Motor symptoms such as hindleg tucking when lifted by the tail were induced in the rat by 5 daily doses of 2 mg each, resulting in brain mercury levels above 8 µg/g cerebral hemisphere or cerebellum, and more than 6 µg/g pons and medulla. Levels of glycine, glutamate, aspartate, and gamma-amino butyric acid (GABA) were unchanged from control levels. In monkeys, single doses of more than 3 mg/animal and 5 doses of 0.75 mg or more produced a neurological degenerative pattern at mercury levels greater than 8 µg/g tissue. GABA was increased and glycine, glutamate, and aspartate were decreased in the brain tissue. Neither single nor repeated doses produced motor responses in the hamster prior to death.

SECTION V

RELATIVE TOXICITY OF VARIOUS METHYL MERCURY COMPOUNDS IN RATS*

INTRODUCTION

Results presented in the previous section indicated that in our hands, the $LD_{50/30}$ for methylmercuric chloride is 8 to 10 mg/kg. Our persual of the literature, which is sparse and not as well documented as it should be, gave most LD_{50} values ranging between 30 and 150 mg/kg for rats - 3 to 15 times our values. Only one report on methyl mercury cyanimide gave a value of 10 mg/kg when dissolved in oil. Another[50] gives 5-6 mg of mercury/kg in mice IV or IP, and probably a similar dose for rats.

Several factors could be responsible for the differences observed: 1) route of administration (we use the intraperitoneal because it gives rapid absorption and mimics the oral route; others have used oral, intravenous and subcutaneous administration), 2) age and sex of the animals and the interval evaluated (important, but not frequently mentioned), 3) vehicle used (i.e., distilled H_2O, saline or oil have all been used, and it may be important), 4) methyl mercury compounds are not very water soluble (some investigators have reported using concentrations greater than we can get in solution). Another factor we have observed is that solutions weaken with time; one reason is interaction with sulfur in the stopper of the bottle. We use fresh solutions to avoid this problem, but some workers may not be aware of this problem.

The subject of this study was the possible effect of the anion. Most investigators have de-emphasized the importance of this possible factor because the anion usually dissociates so that only the methyl mercury ion is left. However, others have discussed possible differences due to degree of dissociation and possible differences of transport of the undissociated molecules.

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*Presented by E. W. Hupp at Texas Academy of Sciences, annual meeting, March, 1976.

The three compounds and their abbreviations used in this study are shown in Figure 3. In a comparative study we used the molecular weights of the compounds to calculate doses that contained equivalent amounts of mercury. Table 7 shows doses based on methylmercuric chloride: the methylmercuric acetate doses were 108.6% of methylmercuric chloride and the methylmercuric hydroxide doses were 92.6% of methylmercuric chloride.

RESULTS

Table 7 presents percent lethality, based on 10 animals per group. The incidence of lethality was greater than that observed following methylmercuric chloride in earlier studies. This may be due to the fact that older solutions may have been used in some of the earlier studies. The earlier LD_{50} based on probit analysis may also have yielded an overestimate. These results do not show a significant difference in lethality in groups exposed to comparable amounts of methyl mercury in the three compounds.

The data presented in Table 8 shows that there also was little difference in the mean survival time of animals exposed to comparable doses of the three compounds, although there was a tendency for the animals exposed to the lower doses of methylmercuric hydroxide to survive somewhat longer than animals receiving the other two compounds. These results show that many of the differences in reported lethal doses in the literature are not due to differences between these methyl mercury compounds.

Figure 3. FORMULAS OF METHYL MERCURY COMPOUNDS USED

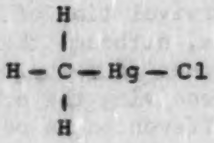
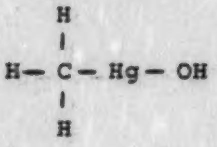
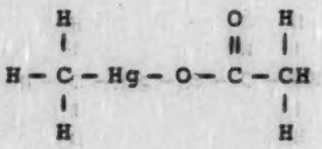
COMPOUND	ABBREVIATION	FORMULA	M.W.
Methyl- Mercuric Chloride	MMC	 $H-\overset{H}{\underset{H}{C}}-Hg-Cl$	251.1
Methyl- Mercuric Hydroxide	MMH	 $H-\overset{H}{\underset{H}{C}}-Hg-OH$	272.7
Methyl- Mercuric Acetate	MMA	 $H-\overset{H}{\underset{H}{C}}-Hg-O-\overset{O}{\overset{\|}{C}}-\overset{H}{\underset{H}{CH}}$	232.6

Table 7. PERCENT OF ANIMALS DEAD FOLLOWING VARIOUS DOSES OF THREE METHYLMERCURIC COMPOUNDS

MMC Dose	Agent		
	MMC	MMA	MMH
mg/kg			
5.00	20	20	10
6.25	80	80	60
7.50	80	60	60
8.75	90	70	80
10.00	80	90	100
Mean	70	64	70

Table 8. SURVIVAL TIME IN DAYS OF RATS EXPOSED TO VARIOUS METHYLMERCURIC COMPOUNDS -- MEAN AND (RANGE)

MMC Dose	Agent		
	MMC	MMA	MMH
mg/kg			
5.00	18.00 (9-27)	5.6 (5-6)	9.0
6.25	2.9 (1-6)	2.6 (1-6)	15.0 (5-31)
7.50	2.2 (1-6)	1.8 (1-4)	2.8 (1-6)
8.75	1.1 (1-2)	2.5 (1-7)	1.1 (1-2)
10.00	1.25 (1-2)	1.1	1.0
20.00	----	1.0	1.0

SECTION VI

RESULTS OBTAINED IN THE MAIN SERIES OF EXPERIMENTS, METHYL MERCURY DOSE ADMINISTERED TO RATS 7 DAYS BEFORE IRRADIATION

GENERAL INTRODUCTION

The animal phase of this series was conducted during the first year of the project. Tissue analysis and interpretation of data continued in later parts of the project. The results of evaluation of various parameters will be presented in separate sections. Brief literature background for each section will be presented in that section.

GENERAL PROCEDURE

The animals used were adult (250 to 300 g) male Sprague-Dawley-derived albino rats. Fifty to 60 animals were assigned to each of the insult combinations listed below:

Combination	Methylmercury mg/100 g body wt.	Radiation kR
1	0	0.0
2	0	4.5
3	0	9.0
4	4	0.0
5	4	4.5
6	8	0.0
7	8	9.0

Radiation was applied with a U. S. Nuclear Corp. Gamma Irradiator at a dose rate of approximately 500 R/min. 7 days after administration of mercury or saline. All animals not receiving irradiation were sham-irradiated. Methyl mercury was injected intraperitoneally. Solutions containing either 3 or 4 mg methylmercuric chloride per ml of physiological saline were used. Only solutions 1 to 3 days old were used, in order to avoid deterioration of the solutions with storage. All animals not receiving methylmercury were injected with physiological saline.

The behavioral and EEG tests, which are described in detail in succeeding sections, were administered during the 6 hours following irradiation. Twenty-four animals per treatment group were used in behavioral tests and 12 other animals were used in the EEG studies. Additional animals were assigned to combinations 6 and 7 to account for mercury-induced lethality. The animals were killed 6 hours after irradiation.

Animals used in behavioral and EEG studies were also used to provide tissue, with an equal number allocated to each of the following analyses:

Analysis	No. Rats from Each Treatment Combination
GABA determination	10
Serotonin determination	10
Mercury distribution studies	10
Histological studies	10

Immediately after killing by decapitation, the heads of the animals to be used for the biochemical studies were frozen in liquid nitrogen. The animals used for histological studies were decapitated, then the brains were removed and placed in fixative immediately. The biochemical and histological analyses were performed as described in the succeeding sections.

A. BEHAVIORAL TESTS

INTRODUCTION

Behavioral changes induced by doses of ionizing radiation have been extensively investigated in adult animals[51] and in progeny of irradiated females.[52] Massive doses have been shown to cause an initial period of performance decrement and incapacitation followed by a temporary recovery, then severe incapacitation and death.[53]

Behavioral changes observed in humans poisoned with methyl mercury compounds[54] include lack of interest, lack of concentration, despondency, anxiety, fits of rage, and change in personality.[55] However, only limited experimental observation of behavioral changes in mercury poisoned animals has been reported.[56]

MATERIALS AND METHODS

The subjects were 243 male Sprague-Dawley rats approximately 300 g in weight at the beginning of the study. One hundred -sixty-one subjects underwent both the open field and sexual behavior tests; the remaining 82 animals served only in the conditioned avoidance study. The animals were housed in groups of 4 to 6 under a reversed 12-12 light-dark cycle with the dark phase beginning at 6 a.m.

The open field consisted of a circular field 81 cm in diameter surrounded by a 33 cm high wall. The field was divided by two concentric circles and lines radiating from the center into 12 truncated triangular-shaped areas surrounding a central circular area. The field was illuminated by a white 100 W bulb suspended 40 cm directly above the center of the field.

Two identical sexual behavior arenas were used in the sexual behavior tests. The arenas were plexiglass-fronted, semicircular enclosures having a height of 28 cm and a radius of 31 cm. The arenas were placed side by side on a table and were illuminated by two red 40 W bulbs suspended approximately 2 m in front of the arenas.

The avoidance conditioning apparatus was a two-compartment Lehigh Valley Electronics plexiglass shuttle box. The 46 x 20 x 18 cm box was divided into two 23 cm long compartments by a partition extending 4.5 cm above the floor. A "Q" light located in the end wall of each compartment 14 cm above the floor provided visual signals and a Sonalert sound source located in the roof of the box above the dividing partition provided an auditory signal. The grid floor of each compartment was connected to an AC shocker that delivered a 1 ma scrambled foot shock. All stimulus events were electromechanically programmed. The shuttle box was contained in a dimly lit room separated from the observer by a one-way glass partition.

All behavior tests were conducted during the second third of the dark phase of the light-dark cycle, and were begun 60-105 minutes after radiation or sham irradiation. Data collection in all tests was conducted under a blind procedure.

The open field test consisted of placing the subject in the central circular area of the field and observing his activity for a period of 2 min. The following measures of emotionality and ambulation were recorded: (a) the number of defecations, (b) the number of areas entered with all four feet (ambulation), and (c) the number of times the animal stood upon his hindlegs (rearings).

Upon completion of the open field test the subject was placed in one of the two sexual arenas and given 5-7 min to adapt to the enclosure. At the end of the adaptation period a stimulus female was introduced to the arena to begin the 30 min sexual behavior test. Stimulus females were ovarectomized at approximately 100 days of age and brought into estrous by intra-muscular injections of 20 μg estradiol benzoate in corn oil followed 36 hr later by 1 mg progesterone. Females were used approximately 10 hr after the progesterone injection.

During the sexual behavior sessions, two subjects were simultaneously tested by a single observer seated approximately 2 m in front of the arenas. The following sexual behavior measures were recorded for each subject: (a) mount frequency (number of mounts without intromission during the 30 min observation period), (b) intromission frequency (number of intromissions during the 30 min observation period), (c) ejaculation frequency (number of ejaculations during the 30 min observation period), (d) mount latency (time from the introduction of female to the first mount), (e) intromission latency (time from introduction of the female to the first intromission), (f) ejaculation latency (time from introduction of female to the first ejaculation), and (g) post-ejaculatory interval (time from first ejaculation to the next mount or intromission).

A conditioned avoidance session was begun by allowing the animal to explore the shuttle box for a 2 min period. At the end of this adaptation period, the first trial of the conditioning sequence was initiated by the onset of the auditory signal and the visual signal in the compartment the subject was exploring. Ten seconds following the onset of the signals, a 10 sec shock was administered through the grid floor of that compartment. The signal remained on during the administration of the shock. The animal made a correct response (avoidance) if he jumped across the partition from the "shock" compartment to the "safe" compartment before the shock was initiated. If the animal failed to make an avoidance response he was allowed to escape the shock by crossing the partition after shock onset. The signals remained on until the end of the 10 sec shock period or until the animal made an avoidance or escape response. At the beginning of each subsequent trial, the compartment containing the subject was designated as the "shock" compartment so that typically each compartment alternated as the "shock" and "safe" side. Each animal was given 100 trials in a single session with a variable 20 sec interval between trials. The number of avoidance responses in 100 trials was recorded for each subject.

RESULTS

OPEN FIELD TESTS

Since only 14.9% of the animals defecated during the open field tests, this variable was not analyzed. The means and standard deviations of each group for the ambulation and rearing scores are presented in Table 9. An analysis of variance for the area and rearing variables yielded F values significant at the F-7.64 $p < .001$ level. The results of the Duncan's Multiple Range Test with unequal n are indicated by the superscript letters in Table 9. It may be noted that the methyl mercury treatment alone had little effect on both measures, as there were no differences between the two mercury groups and controls. Both doses of radiation alone significantly suppressed both activity measures in relation to the controls, with the larger dose producing the greater effect. In all cases, the effect of the co-insult was less than that of the same dose of radiation alone, but due to the large variation within samples, these differences were not significant at the $p=.05$ level.

SEXUAL BEHAVIOR TESTS

The percentage of animals in each group exhibiting at least one mount, intromission, and ejaculation during the 30 min observation period is presented in Table 10. A Chi-square test of independence was employed to determine if the proportion of animals exhibiting a particular response was the same for all treatments. This comparison was significant at the .001 level for each of the three variables. Although neither dose of methyl mercury presented alone produced a large reduction in the proportion of animals exhibiting each response, both levels of radiation alone severely reduced sexual activity, with the high radiation level almost completely abolishing this behavior. As was the case with the open field measures, co-insult treatments were less disruptive of sexual activity than the same doses of radiation alone, although these differences were not significant.

The means and standard deviations of the number of mounts, the number of intromissions, and the number of ejaculations occurring during the 30 min session for each treatment level are displayed in Table 11. The analysis of variance for the variables mounts, intromissions, and ejaculations yielded F values significant at the $p < .001$ level.

The results of the Duncan's Test for each of these variables are also presented in Table 11. The controls exhibited the greatest number of mounts, intromissions, and ejaculations, and differed from all other groups on the latter two criteria. As was generally the case with the previously mentioned behavior patterns, 9 kR had a greater effect in depressing sexual behavior than 4.5 kR, and the co-insult was less

Table 9. MEANS AND STANDARD DEVIATIONS OF OPEN FIELD MEASURES OF RATS TREATED WITH METHYL MERCURY AND/OR GAMMA RADIATION

Methyl Mercury Dose (mg/kg body weight)	Radiation Dose (kR)	Number of Animals	Number of Areas Entered	Number of Rearings
0.0	0.0	22	23.95[a] ±8.73	7.77[a,b] ±5.42
0.0	4.5	22	17.68[b,c] ±5.65	4.00[c] ±3.19
0.0	9.0	21	10.76[d] ±9.03	2.57[c] ±2.78
4.0	0.0	22	23.14[a] ±9.67	8.68[a] ±6.62
4.0	4.5	22	17.86[b,c] ±7.40	5.14[b,c] ±2.95
8.0	0.0	26	20.12[a,b] ±9.05	7.73[a,b] ±5.17
8.0	9.0	26	13.69[c,d] ±6.97	3.88[c] ±4.02

[a,b,c,d] Means in the same column with the same superscript do not differ at the 5% level as tested by Duncan's test.

Table 10. PROPORTION OF ANIMALS RECEIVING METHYL MERCURY AND/OR GAMMA RADIATION WHO ACHIEVED AT LEAST ONE MOUNT, INTROMISSION AND EJACULATION

Methyl Mercury Dose (mg/kg body weight)	Radiation Dose (kR)	Number of Animals	Mounts	Intro-Missions	Ejacula-tions
0.0	0.0	22	0.95	0.95	0.91
0.0	4.5	22	0.41	0.32	0.32
0.0	9.0	21	0.10	0.00	0.00
4.0	0.0	22	0.82	0.59	0.59
4.0	4.5	22	0.59	0.41	0.41
8.0	0.0	26	0.73	0.58	0.58
8.0	9.0	26	0.31	0.15	0.15

Table 11. MEANS AND STANDARD DEVIATIONS OF SEXUAL BEHAVIOR MEASURES OF RATS TREATED WITH METHYL MERCURY AND/OR GAMMA RADIATION

Methyl Mercury Dose (mg/kg body weight)	Radiation Dose (kR)	Number of Animals	Number of Mounts	No. of Intromissions	No. of Ejaculations
0.0	0.0	22	20.77^{a} ± 8.56	21.45 ±13.99	1.91 ±0.92
0.0	4.5	22	$7.95^{c,d}$ ±11.67	$5.59^{b,c,d}$ ± 9.37	$0.73^{a,b}$ ±1.16
0.0	9.0	21	1.14^{d} ± 5.01	0.71^{d} ± 3.28	0.0^{c} ±0.0
4.0	0.0	22	$17.05^{a,b}$ ±14.27	13.77^{a} ±11.27	1.23^{a} ±1.11
4.0	4.5	22	$11.09^{b,c}$ ±11.18	$8.36^{a,b,c}$ ± 9.34	$0.73^{a,b}$ ±0.93
8.0	0.0	26	$15.35^{a,b}$ ±12.61	$11.27^{a,b}$ ±11.03	$1.04^{a,b}$ ±1.04
8.0	9.0	26	2.62^{d} ± 5.17	$2.81^{c,d}$ ± 7.56	$0.35^{b,c}$ ±0.89

[a,b,c,d] Means in the same column with the same superscript do not differ at the 5% level of probability, as tested by Duncan's test.

effective, though not significantly so, in depressing activity than the same dose of radiation delivered alone.

Since the analysis presented in Table 11 included those animals failing to exhibit the various sexual activities (i.e., having a zero frequency for the category), additional comparisons were made to determine if the behavior of the sexually active animals in the experimental conditions was different from that of the active controls. An animal failing to engage in a particular activity was therefore excluded from the comparsion for that sexual behavior category, thus reducing the number of subjects in each condition included in the comparison. Although two animals in the 9 kR alone condition were observed to mount, this condition was excluded from these analyses because none of these animals were observed to achieve an ejaculation. The means and standard deviations of the frequency and latency categories for the remaining subjects are presented in Table 12. The mean number of intromissions required to reach the first ejaculation for each condition is also included in this table. The analyses of variance for the number of intromissions ($F_{5,68}$=1.14), the number of ejaculations ($F_{5,62}$=1.00), and the number of intromissions to the first ejaculation ($F_{5,62}$=1.35) categories yielded nonsignificant outcomes ($p < .05$). The analysis of the number of mounts variable was marginally significant ($F_{5,82}$=2.53; $p < .05$), an effect apparently due to the low mean mount frequency of the 8 mg/kg, 9 kR animals. The four latency measures for the six groups were compared by means of the Kruskal-Wallace analysis of variance by ranks. The comparisons for the mount latency (H_5=1.13), intromission latency (H_5=10.94), and postejaculatory interval (H_5=5.61) failed to reach significance ($p < .05$); however, the analysis of the ejaculation latency variable was significant (H_5=11.35).

CONDITIONED AVOIDANCE TEST

The analysis of variance (F=5.56; $p < .001$) and the subsequent Duncan's Test indicated that the controls exhibited the greatest number of correct conditioned avoidance responses (see Table 13), a finding consistent with the open field and sexual behavior tests. The group receiving 8 mg mercury and no radiation, however, did not differ significantly from the controls but was considerably lower in the correct number of responses. All other treatment groups were significantly lower in performance than these two groups, but did not differ significantly from each other.

DISCUSSION

In all frequency measures except the number of rearings in the open field, the control animals exhibited the greatest amount of activity. Methyl mercury injections, however, did not significantly affect the open field behavior measured in the present study, a finding that is surprising in light of previous open field studies which indicated methyl mercury injections reduced open field activity. Both levels of

Table 12. MEANS AND STANDARD DEVIATIONS OF SEXUAL BEHAVIOR MEASURES OF SEXUALLY ACTIVE RATS TREATED WITH METHYL MERCURY AND/OR GAMMA RADIATION

Methyl Mercury Dose	Radiation Dose	No. of Mounts	Mount Latency	No. of Intromissions	Intromission Latency	No. of Ejaculations	Ejaculation Latency	Post-Ejac. Interval	No. of Intromiss. to First Ejaculation
0.0	0.0	21.76 ± 7.37	119.48 ±125.37	22.48 ±13.48	240.05 ±219.83	2.10 ±0.72	748.90 ±228.43	297.30 ±128.77	11.55 ± 5.22
0.0	4.5	19.44 ±10.28	128.44 ±165.29	17.57 ± 7.27	161.29 ±184.92	2.29 ±0.76	498.57 ±256.40	329.29 ± 35.50	7.70 ± 3.59
4.0	0.0	20.83 ±12.96	199.61 ±303.25	21.64 ± 4.67	164.36 ±190.27	2.08 ±0.49	643.46 ±195.53	293.85 ± 89.02	12.76 ± 4.08
4.0	4.5	18.76 ± 7.91	179.23 ±124.71	15.33 ± 7.06	410.00 ±454.38	1.78 ±0.41	929.22 ±272.18	363.00 ± 96.65	11.33 ± 3.43
8.0	0.0	21.05 ± 9.75	125.79 ±170.45	18.31 ± 8.07	289.63 ±293.78	1.80 ±0.69	824.47 ±404.85	285.79 ±110.34	11.27 ± 4.88
8.0	9.0	8.50 ± 6.19	412.50 ±579.03	18.25 ±10.05	277.75 ±399.02	2.25 ±0.96	657.00 ±544.95	285.33 ±144.95	9.25 ± 0.96

Table 13. MEANS AND STANDARD DEVIATIONS OF NUMBER OF CORRECT RESPONSES ON CONDITIONED AVOIDANCE TEST

Methyl Mercury Dose (mg/kg body weight)	Radiation Dose (kR)	No. of Animals	Number of Correct Responses
0.0	0.0	11	25.73[a] ±21.54
0.0	4.5	11	6.73[b] ± 9.97
0.0	9.0	11	5.73[b] ± 6.89
4.0	0.0	10	7.0[b] ± 5.87
4.0	4.5	10	6.6[b] ± 8.50
8.0	0.0	14	17.43[a] ±16.41
8.0	9.0	15	2.6[b] ± 4.57

[a,b]Means with the same superscript do not differ at the 5% level of probability as tested by Duncan's test.

gamma radiation significantly suppressed open field activity as indicated by the areas entered and rearings variables.

Radiation also had more of an effect on sexual behavior measures than did mercury injections; in the 9 kR alone condition, sexual activity was almost completely abolished. In the number of mounts variable, the animals in both mercury conditions, though lower, were not significantly different from controls. Mercury-injected rats, however, achieved fewer intromissions and ejaculations than did control animals, although there was no difference between the low and high dose levels on these variables. The comparisons of the sexually active animals in the experimental groups with the active controls pointed to a remarkable similarity of behavior on both frequency and latency variables; those animals that were sexually active were generally not distinguishable from animals in other treatment levels. These findings suggest that the significant treatment effects noted in Table 11 were principally due to the almost complete suppression of sexual behavior in some subjects and point to the wide variability of these animals in susceptibility to radiation, mercury poisoning, and co-insults of these agents.

The most interesting findings of the present study concerned the comparisons of the co-insult conditions with the radiation alone groups. In the open field and sexual behavior tests, those animals receiving the co-insult generally performed better than those receiving the same dose of radiation alone. While these differences were not significant, their consistency throughout the various tests suggests that methyl mercury in the co-insult groups inhibited the effect of radiation upon the nervous system as measured by the behavioral tests used in this study.

SUMMARY

Behavior of all subjects was measured by open field, sexual behavior, and conditioned avoidance tests beginning 60-105 minutes after radiation or sham irradiation. There was a tendency for the co-insult treatment groups to show fewer disruptive effects than were shown by the radiation alone groups. Although the conditioned avoidance test yielded reliable differences between controls and all experimental conditions except for the 8 mg MMC/kg treatment, the test did not distinguish between insult levels. The open field and sexual behavior tests showed minimal effects of mercury alone and significant effects of radiation alone, suggesting that MMC in the co-insult groups inhibited the effect of radiation on the nervous system.

B. EEG Analyses

Electroencephalograms were made on 12 animals in each of the seven treatment groups 4 to 6 hours after the completion of irradiation or sham-irradiation. The frequency and amplitude of the EEG was measured in five or more 5-second intervals for each animal. The results are summarized in Table 14. Changes revealed by this quantitative analysis are limited. Radiation alone increased the amplitude of the EEG and caused a slight decrease in frequency. Mercury treatment alone had little apparent effect, no changes in amplitude were produced, but the 4 mg/kg dose appeared to decrease the frequency while 8 mg/kg appeared to increase the frequency of the EEG. In the low-level co-insult (4 mg/kg mercury and 4.5 kR) mercury apparently had no effect on the changes induced by irradiation, since the means were very similar. The effect of the mercury treatment in the high level co-insult (8 mg/kg mercury and 9 kR) is not clear-cut, the amplitude was reduced to a value intermediate between 9 kR alone and control, but the frequency was reduced more by the co-insult than by radiation alone.

A subjective examination of the recordings also revealed limited changes. Striking changes such as those elicited by a variety of stimuli, including irradiation[57] were not observed. However, some increase in "spiking" previously described by Speck[58] appeared to occur in the 9 kR group; these changes were less evident in the high level co-insult group.

C. DISTRIBUTION OF METHYL MERCURY IN RAT BRAINS

METHODS

The rats were killed by decapitation 6 hours after radiation. The heads were immediately dropped into liquid nitrogen and frozen. They were then stored at -28°C until the brain parts were dissected. The brain was kept frozen by dissecting on a glass surface cooled by dry ice. Separate brain parts were placed in pre-weighed vials, weighed and then again frozen and stored at -28°C in the storage vials until ready for digestion. The same vial was used for weighing, storage and digestion. For some of the samples nitric acid digestion[59] was used to prepare the samples for total mercury analysis by cold vapor atomic absorption. For the remainder of the samples Thorpe's[60] sulfuric acid-permanganate digestion was used.

RESULTS

The samples in series A,B,C, that had received no dose of mercury showed some residual levels of mercury even after reagent blank correction. This residual level was higher in the case of those samples digested in nitric acid than those digested in sulfuric acid-permanganate. In neither case, however, did the samples show any difference dependent on brain area or radiation treatment. Thus, these background levels were averaged for each method, and subtracted from the individual mercury determinations in the D,E,F,G, series animals.

Table 14. FREQUENCY AND AMPLITUDE OF THE ELECTROENCEPHALOGRAM OF RATS EXPOSED TO RADIATION AND/OR METHYL MERCURY

	Treatment		Electroencephalogram	
Group	Rad. kR	Methyl Mercury mg/kg	Frequency Cycles/sec.	Amplitude uv.
A	0.0	0.0	10.9	73
B	4.5	0.0	9.3	80
C	9.0	0.0	9.9	90
D	0.0	4.0	9.2	70
E	4.5	4.0	9.2	80
F	0.0	8.0	11.7	73
G	9.0	8.0	9.1	80

The results of the total mercury analysis of the brains from rats dosed with two levels of methyl mercury and two levels of radiation are presented in Table 15.

Methyl mercury concentrations were higher in the cerebellum than the pons-medulla or cerebral cortex regardless of mercury or radiation dosage. Data indicates that the pons-medulla and cerebral cortex absorbed about the same level of mercury for any one treatment. The samples that had received 8 mg/kg dose of mercury (series F & G) had greater mercury concentrations by factors of 2 to 4 over those samples that had received only the 4 mg/kg dose of mercury (series D & E).

Radiation appeared to decrease the content of methyl mercury in all brain sections. This decrease does appear to be related to radiation dosage. The pons-medulla samples of series E appear to be an exception to the above observations. There was no data to indicate that the analysis of these samples was at fault. No explanation can be given for the anomalous results for these samples. These total mercury analyses can be taken as total methyl mercury since experimentation demonstrated that most of the mercury present was in the organic form. Whole brains from two animals were homogenized and 5 aliquots from each brain were analyzed for total mercury and inorganic mercury by amodification of the method of Magos and Clarkson.[61] By substraction the organic mercury, presumably methyl mercury, was calculated. The results are given in Table 16.

The results of the mercury analyses provide a basis to relate the effects observed by the various measures to mercury concentrations in the brain responsible for these effects. They also demonstrate that most of the methyl mercury (over 90%) injected that remains in the brain 7 days later is still in the organic form.

D. NEUROTRANSMITTER ANALYSIS

The serotonin (5-HT) and norepinephrine (NE) levels in five brain areas of the rat were determined using the technique of Maickel et al.[62] The animals were injected with methyl mercury chloride (MMC), then seven days later they were irradiated and six hours later sacrificed by decapitation. Immediately after decapitation the heads were dropped into liquid nitrogen and kept at -70°C until the time of analysis. The brain areas used for analysis were the cerebral cortex, thalamus, midbrain, pons-medulla and cerebellum. The animals were divided into seven groups: control, 0.4 mg/kg MMC only 0.8 mg/kg MMC only, 4.5 kR only, 9.0 kR only, 0.4 mg/kg MMC plus 4.5 kR and 0.8 mg/kg MMC plus 9.0 kR.

Table 17 shows the results of these treatments on the norepinephrine levels in the various rat brain areas. A consistent pattern is seen in those animals treated with MMC. There is a decrease in NE with the administration of 0.4 mg/kg MMC and an increase at 0.8 mg/kg MMC over the 0.4 mg/kg level. Alteration of the NE levels by irradiation presents

Table 15. MERCURY CONTENT (μg/g brain) OF VARIOUS BRAIN AREAS
OF RATS DOSED WITH METHYL MERCURY

Treat. Group	D	E	F	G
Methyl Mercury Dose mg/kg	4.0	4.0	8.0	8.0
Radiation kR	0.0	4.5	0.0	9.0
Brain Area:				
Cerebral Cortex	0.703±0.132[a] (13)[b]	0.569±0.195 (15)	1.516±0.250 (14)	1.191±0.248 (15)
Cerebellum	0.846±0.134 (14)	0.687±0.147 (14)	2.185±0.733 (17)	1.537±0.362 (15)
Pons-medulla	0.431±0.118 (13)	0.725±0.174 (13)	1.676±0.359 (17)	0.935±0.222 (15)

[a] mean standard deviations

[b] number in brackets denotes number of samples represented in mean

Table 16. TOTAL, INORGANIC AND ORGANIC MERCURY CONTENT OF HOMOGENATE OF WHOLE BRAIN OF RATS DOSED WITH METHYL MERCURY

Dose	Sample	Total Hg (µg/g)	Inorganic Hg (µg/g)	Organic Hg	
				g/g	% of total
4 mg/kg	1	0.775	0.072	0.703	90.7
	2	0.834	0.091	0.753	90.6
	3	0.647	0.055	0.592	91.5
	4	0.806	0.061	0.745	91.2
	5	0.688	0.049	0.639	92.9
8 mg/kg	6	1.421	0.127	1.294	91.1
	7	1.307	0.112	1.195	91.4
	8	1.529	0.123	1.406	91.3
	9	1.493	0.142	1.351	90.5
	10	1.602	0.151	1.451	90.6
				avg	91.2

Table 17. NOREFINEPHRINE ANALYSIS

(μg NE/g tissue)

Treatment	Cortex	Cerebellum	Midbrain	Pons-Medulla	Thalamus
0.0 kR-0.0 mg MMCl	1.41±0.376	0.679±0.151	2.75±0.493	1.24±0.311	2.18±0.522
0.0 kR-0.4 mg MMCl	0.714±0.144	0.364±0.0856	1.46±0.258	1.01±0.182	0.933±0.184
0.0 kR-0.8 mg MMCl	1.12±0.222	0.477±0.101	1.54±0.405	1.16±0.125	2.62±0.434
4.5 kR-0.0 mg MMCl	0.649±0.0575	0.468±0.114	1.48±0.517	1.25±0.185	2.14±0.758
4.5 kR-0.4 mg MMCl	0.656±0.0923	0.322±0.0631	2.34±0.565	0.896±0.155	2.00±0.249
9.0 kR-0.0 mg MMCl	0.918±0.112	0.350±0.0463	2.49±0.795	1.16±0.222	2.59±0.396
9.0 kR-0.8 mg MMCl	0.995±0.242	0.482±0.0897	2.69±0.637	0.891±0.244	1.61±0.193

Table 17. SEROTONIN ANALYSIS

(μg 5HT/g tissue)

Treatment	Cortex	Cerebellum	Midbrain	Pons-Medulla	Thalamus
0.0 kR-0.0 mg MMCl	0.442±0.0237	0.209±0.00900	0.868±0.0685	0.393±0.150	0.657±0.309
0.0 kR-0.4 mg MMCl	0.170±0.0372	0.0512±0.0978	0.188±0.0386	0.127±0.0210	0.142±0.0301
0.0 kR-0.8 mg MMCl	0.266±0.0397	0.0922±0.0182	0.473±0.116	0.178±0.0215	0.366±0.0770
4.5 kR-0.0 mg MMCl	0.207±0.0491	0.171±0.0280	0.424±0.0985	0.212±0.0415	0.573±0.0647
4.5 kR-0.4 mg MMCl	0.162±0.0147	0.0837±0.0112	0.594±0.120	0.181±0.0311	0.278±0.466
9.0 kR-0.0 mg MMCl	0.157±0.0251	0.0656±0.00835	0.327±0.0315	0.188±0.0468	0.379±0.0428
9.0 kR-0.8 mg MMCl	0.156±0.0166	0.0574±0.00598	0.417±0.0887	0.183±0.0523	0.216±0.0308

a more complex picture: the cerebral cortex and midbrain show a decrease at 4.5 kR and a rise at 9.0 kR over the 4.5 kR levels. The changes in the NE levels of animals given the co-insult do not follow a distinct pattern. Significant changes are seen in the cerebral cortex and cerebellum at 4.5 mg/kg MMC plus 4.5 kR treatment.

Table 17 also shows the effects of the treatments on the 5-HT levels in the various brain areas of the rat. The results seen in all the treatment groups follow a distinct pattern. Those treated with MMC alone show a decrease at 0.4 mg/kg and a rise over the 0.4 mg/kg at 0.8 mg/kg. Animals treated with x-irradiation show a decreasing level of 5-HT with increasing doses of radiation. The pattern seen in the animals receiving the co-insult is similar to that of the irradiated animals; for this reason it may be that the x-irradiation is the predominate insult in these cases. In general our levels of NE are higher than those reported in the literature for untreated animals and somewhat lower than the values reported for 5-HT.

Table 18 summarizes the percent change from untreated animals in the levels of NE and 5-HT in the various brain areas. The lowest percent changes in NE occurs in the cerebral cortex, cerebellum and midbrain. The percent changes in the 5-HT levels seem to be uniformly large in all areas of the brain. In general the serotonin seems to be affected by all treatments to a greater extent than the NE.

The effects of MMC on the transmitter levels suggests that the mode of action may invlove two mechanisms. Both NE and 5-HT show a decrease at 0.4 mg/kg MMC with a rise in the levels at 0.8 mg/kg. If one considers the possible causes for a decrease in transmitter levels from exposure to MMC two aspects may be considered. First is that there is a decrease in the availability of precursors to synthesize the transmitters, and the second that there may be a reduction in enzymes available for synthesis. Yoshino et al.,[63] found that MMC also inhibits protein synthesis. The inhibition of protein synthesis should reduce the levels of enzymes available for synthesis. Either of these findings might explain the reduction in transmitter levels brought about by methyl mercury.

A number of workers have reported a decrease in the brain serotonin content resulting from x-irradiation.[64] No reports are available for the effect of x-irradiation on NE brain levels.

The effect of the co-insult on the transmitter levels clearly does not seem to be additive for either NE or 5-HT. Data obtained for 5-HT exposed to co-insult suggests that the pattern of change is similar to x-irradiated animals, but not the methyl mercury treated animals. All of the treatments have a considerable effect on the transmitter levels in most brain areas. Of the two transmitters studied, 5-HT has been found to be more sensitive to the various treatments. Because of the effects obtained on levels of both transmitters, it is suggested that possible mechanisms of action and correlations to the neurological changes be explored.

Table 18. PERCENT CHANGE FROM CONTROL IN NEUROTRANSMITTERS RESULTING FROM VARIOUS INSULTS

Norepinephrine Analysis

Treatment	Cortex	Cerebellum	Midbrain	Pons-Medulla	Thalamus
0.0kR-0.4 mg MMCl	-49.4	-46.4	-46.9	-18.5	-57.2
0.0kR-0.8 mg MMCl	-20.6	-29.7	-44.0	-06.4	+20.2
4.5kR-0.0 mg MMCl	-54.0	-31.1	-46.2	+00.8	-01.8
4.5kR-0.4 mg MMCl	-53.5	-52.6	-14.9	-27.7	-08.3
9.0kR-0.0 mg MMCl	-34.9	-48.4	-09.4	-06.4	+18.8
9.0kR-0.8 mg MMCl	-29.4	-29.0	-02.2	-33.3	-26.1

Serotonin Analysis

Treatment	Cortex	Cerebellum	Midbrain	Pons-Medulla	Thalamus
0.0kR-0.4 mg MMCl	-61.5	-75.5	-78.3	-67.7	-78.4
0.0kR-0.8 mg MMCl	-39.8	-55.8	-45.5	-54.7	-44.3
4.5kR-0.0 mg MMCl	-53.2	-18.2	-51.2	-46.0	-12.8
4.5kR-0.4 mg MMCl	-63.3	-59.9	-31.6	-53.9	-57.7
9.0kR-0.0 mg MMCl	-64.4	-68.6	-62.3	-52.7	-42.3
9.0kR-0.8 mg MMCl	-64.7	-72.5	-52.0	-53.4	-67.1

E. THE EFFECT OF METHYLMERCURIC CHLORIDE AND IONIZING RADIATION ON THE RAT BRAIN*

INTRODUCTION

Autoradiographic studies of mercury poisoning have shown preferential accumulation of the mercury within the cytoplasm of large neurons, particularly those in the cerebellum and brain stem.[65] The cerebellar changes seen in all species studied by Cavanagh and Chen[66] have been confined to granular cells, either as diffuse loss or in focal discrete areas. Their findings confirmed the original observation of Hunter et al.[67] that in the rat degenerative lesions were almost entirely confined to the primary sensory neurons of dorsal root and trigeminal ganglia, and to the granular cells of the cerebellar cortex. The majority of the literature reviewed coincided in the finding that the cerebellum was probably the most affected area of the central nervous system. More specifically, the granular layer was impaired more than the other cell layers.[68]

Massive doses of ionizing radiation regularly and promptly bring about characteristic morphologic alterations in certain neural tissues and in the mesenchymal structures in and around the brain.[69] It is well established that exposure of brains of cats, monkeys, rabbits, guinea pigs, mice, and rats to massive doses of ionizing radiation is followed promptly by nuclear shrinkage and hyperchromatism in many of the cerebellar granular cells.[70] These lesions were found with increasing frequency and intensity after doses of 5,000 and 10,000 R, and the cytologic changes were well established within 2 hours after radiation.[71]

The literature reviewed reported that both agents--methylmercuric chloride and ionizing radiation--have a more detrimental effect on the cerebellum than other areas of the central nervous system when administered in a dosage large enough to produce these cytological changes. However, as of yet it is not known what, if any, effect these two insults have on each other.

The present study was designed to evaluate effects of the co-insult at the light microscopy level.

PROCEDURE

Ten rats from each treatment group described previously were used for histological observations. Immediately after decapitation, the whole brain was removed from the cranial cavity by careful dissection with bone clippers and various other dissection instruments. The whole brain was fixed in 10% buffered formalin for 72 hours, then dehydrated in a series of graded alcohols using a tissumaton (Fisher Scientific Apparatus) cycle of 18 hours. The brains were embedded in paraffin (61°C) and cut sagitally at 5 microns with an A-O Spencer "820" microtome. Three sections were mounted on each slide and then stained with hematoxylin-eosin.

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*Portions of these data were submitted by Mitzi Short Thrutchley in partial fulfillment of the requirements for the degree of Master of Science in Biology in the graduate school of Texas Woman's University.

Of the slides prepared, five suitable sections of the brain were selected from each group. The same sections were also used for cell counting in the cerebellum. The different cell types were counted in an approximate 5 X 5 micron square in the central lobule of the cerebellar hemisphere. The counts were averaged to obtain an estimate of the packing density of granule cells.

All sections were analyzed with a Reichert "Zetopan" research microscope. The brain areas examined were the cerebrum, pons, medulla, and cerebellum. Photographs (35 mm) were taken of each group representative of the areas affected. A Kodak high contrast copy film (Eastman Kodak Co., Rochester, N.Y. 14650) was used because of its extreme resolution.

Cell counts of the cerebellar cells were taken of five different sections (separate animals) of each treatment group, and were counted using a laboratory counter (Clay Adams Division of Becton, Dickinson, and Co., Parsippany, N.J.) to determine if there was a loss of cells due to the different treatments. Another cell count was taken on the granule cells to count normal versus pyknotic, and to establish if pyknosis was intermediate or complete. These cell counts were done on 5 animal sections in each treatment group using a set number of 200 cells.

RESULTS

HISTOLOGICAL OBSERVATIONS

The gross appearance of the cerebellum, cerebrum, pons, and medulla in the control group that received sham injection and sham irradiation appeared normal when examined with the light microscope. In the sham injected and 4.5 kR group there were a few pyknotic granule cells scattered throughout the granular layer of the cerebellum, though not as many were viewed here as in the higher radiation dosage group. The cerebrum, pons, and medulla appeared free from change in any way.

In the animals that received sham injection and 9 kR, the cells of the granular layer of the cerebellar cortex were altered notably by pyknosis, thus implicating a direct effect of irradiation on nerve cells. These pyknotic cells were present in almost all the cerebellar lobes, but were scattered randomly rather than dense. As a rule the pyknotic cells were isolated, but now and then were present in groups.

The nuclei of these pyknotic cells were greatly reduced in size and intensely hyperchromatic. The neuronal changes consisted mainly of pyknosis and karyorrhexis of the cerebellar internal granular layer, especially beneath the Purkinje cells.

No changes were found in Golgi cells. Purkinje cells appeared normal. Some Purkinje cells were dark and others showed cytoplasmic chromatolysis and vacuolation, but since these changes were also in sections from control animals, they were interpreted as artificats due to the type of immersion fixation. In the molecular layer few changes were seen, although some basket cells looked like empty shells and some nuclei were rather poor in chromatin. Occasionally pyknotic nuclei cells were seen. No lesions were detected and no perivascular cuffing by leukocytes were observed in the cerebellar cortex. The presence of many red blood cells was evident.

The three other areas investigated--cerebrum, pons, and medulla--were not affected by the large radiation dose. There was a possible loss of granule cells, but as far as could be determined, it was not statistically significant. No structural changes were seen in the axons of the granule cells.

The changes in morphology of groups given 4 mg/kg of methylmercuric chloride (MMC) and then sham irradiated were slight. Only some granule cells appeared pyknotic and these were very minimal. The Purkinje cells appeared normal, as did the molecular layer. As with the previous treatment groups, the cerebrum, pons, and medulla seemed free from any cytological changes.

The smaller co-insult dosage group that was treated with 4 mg/kg MMC and then given 4.5 kR was affected very little histologically. In less than half of the slides analyzed were there any alterations, and these appeared in the cerebellar cortex, usually in the granular layer. These granular cells appeared to be in an intermediate stage undergoing cell pyknosis, but had not completely reached this stage of cell death. In this treatment group the other three brain areas remained normal with no indication of alteration. It was interesting to note that in this group with the smaller does of the insults, the combined agents produced less cell damage than either the smaller of the individual mercury insults or the smaller individual radiation insult.

Histologically, the group receiving the 8 mg/kg MMC and sham irradiation has some noted alterations and these were only found in the cerebellum. Pyknosis of isolated granule cells in the internal layer were randomly scattered throughout the cerebellum. By comparison with the large radiation dosage, this damage was not as pronounced as the radiation damage. The Purkinje cells were observed to be normal as were all of the other cell types in the cerebellum. It is interesting to note that although the degeneration was much more extensive in the granular cell layer, more mercury was localized in the Purkinje cells by autoradiographic studies done by Cassano et al.[72]

The final treatment group that received the larger doses of the co-insult, 8 mg/kg MMC and 9 kR, was surprisingly less affected than the large mercury-treated group. As usual, the only portion of the brain affected was the cerebellum and more specifically, the granular layer. The destruction in this layer consisted of only intermediate pyknosis that was previously described in the smaller co-insult group. The Purkinje cells showed no sign of degeneration, nor did any of the other cerebellar cell types.

In summary, the degree of granule cell degeneration in terms of treatment groups can be seen in Table 19.

BRAIN CELL COUNT FOR POSSIBLE LOSS OF CELLS

Cell counts were taken from the anterior lobe area in the central lobule of the cerebellum. The various brain cells counted consisted of Purkinje, basket, granule, molecular layer cells, and granular cells in the molecular layer. In the granular layer of the cerebellum, only a sample number of granule cells were counted due to the vast numbers. This was done by counting 5 oil fields of approximately 0.5 mm square, so the number of granule cells in this study was only a representative sample rather than the total number. The remaining four cell types were actual numbers of cells in one lobe (anterior) that were counted on 5 different animals and then averaged. The observed and expected cell counts for the possible loss of cells were represented in Table 20. The experiment tested the hypothesis that there was no difference between the various treatment groups on the specific cell type. Therefore, a chi-square test was calculated for each of the five different cell types and can be seen in Table 21.

At 6° of freedom, the tabulated chi-square (X^2) value at the 0.05 level is 12.59. The expected cell count would be an average of the total number of cells counted, since the hypothesis is testing no differences. Since X^2 of the Purkinje cells was less than 12.59, it was concluded that there was no difference between treatment groups in terms of loss of cells. However, in all of the other types of cells, the calculated X^2 was larger than the tabulated X^2 value, indicating that there was a difference in number of cells between the different treatment groups.

Another cell count was made in the cerebellum on normal versus pyknotic granular cells. The degree of pyknosis was also indicated as either intermediate or complete pyknosis. A set number of 200 cells was counted so cell density was not involved. Five sections of each treatment group were counted, and their mean and standard deviation presented in Table 22.

Table 19. DEGREE OF GRANULE CELL DEGENERATION FROM LARGEST TO SMALLEST

Treatment Group
Sham injected and 9.0 kR
8.0 mg/kg MMC and sham irradiated
8.0 mg/kg MMC and 9.0 kR
Sham injected and 4.5 kR
4.0 mg/kg MMC and 4.5 kR
4.0 mg/kg MMC and sham irradiated
Sham injected and sham irradiated

Table 20. OBSERVED AND EXPECTED CELL COUNTS FOR POSSIBLE LOSS OF CELLS

Cell Type		Treatment						
	Control	4.5 kR	9.0 kR	4mg/kg	4.5 kR + 4mg/kg	8mg/kg	8mg/kg + 9.0 kR	Total
Purkinje	*O= 54.0	50.0	45.2	50.6	60.6	44.2	48.2	353.0
	E= 50.4	50.4	50.4	50.4	50.4	50.4	50.4	
Basket	O=106.0	103.2	146.8	142.6	151.0	140.4	137.6	927.6
	E=132.5	132.5	132.5	132.5	132.5	132.5	132.5	
Granule	O=526.6	502.4	395.2	425.6	462.6	400.8	411.4	3124.6
	E=446.4	446.4	446.4	446.4	446.4	446.4	446.4	
Molecular Layer	O=463.4	532.0	451.0	442.2	449.8	375.2	385.6	3098.2
	E=442.6	442.6	442.6	442.6	442.6	442.6	442.6	
Granule in Molecular	O=142.2	130.2	118.0	134.0	156.0	109.8	107.0	897.2
	E=128.2	128.2	128.2	128.2	128.2	128.2	128.2	

*O=observed; E=expected

Table 21. CHI-SQUARE VALUES FOR CELL COUNTS

Cell Type	Chi-Square Value
Purkinje cells	3.72
Basket cells	17.29
Granule cells	36.27
Molecular layer cells	36.83
Granule in molecular layer	14.81

Table 22. MEAN* CELL COUNTS AND STANDARD DEVIATION FOR PYKNOTIC GRANULE CELLS

Treatment Group	Degree of Pyknosis		
	None	Intermediate	Complete
Control (A)			
Mean	200.00		
**St. Dev.	0.0		
4.5 kR (B)			
Mean	190.60	9.40	
St. Dev.	2.65	2.65	
9.0 kR (C)			
Mean	180.20	10.80	8.40
St. Dev.	3.54	3.60	4.13
4.0 mg/kg (D)			
Mean	194.20	6.00	
St. Dev.	1.67	1.67	
4.5 kR + 4.0 mg/kg (E)			
Mean	192.20	7.80	
St. Dev.	1.72	1.72	
8.0 mg/kg (F)			
Mean	184.20	9.60	6.50
St. Dev.	2.79	2.85	3.84
9.0 kR + 8.0 mg/kg (G)			
Mean	185.40	14.60	
St. Dev.	2.87	2.87	

*Mean of 5 sections
**St. Dev. = Standard Deviation

DISCUSSION AND CONCLUSIONS

RADIATION INSULT

The extreme radiosensitivity of the granular layer of the cerebellar cortex has been well established in this study. The granule cell was, in fact, the most radio-vulnerable cerebellar element. Purkinje cells suffered much less alteration than granule cells, and the incidence of their involvement was also lower in this and previous studies.

Low radiosensitivity of Purkinje cells as compared with granule cells was recognized, for example, from the observation by Hager et al.[73] that Purkinje cells in the hamster were free from change at 22 hours following exposure to 40 kR x-irradiation, at a time when granule cells were shruken and showed clumping of nucleoplasm and at a time when capillaries exhibited endothelial-cell swelling. The present study used only 9 kR, and these degenerative changes were observed in the granule cells while the Purkinje cells remained unaltered.

There is still little knowledge of the earliest histopathologic and histochemical changes occurring in the cerebellum following irradiation or in the sequence in which the changes develop. Histochemical investigations by Kimeldorf and Hunt[74] showed that during early radiation damage of cerebellar tissue following high x-irradiation dose, changes occurred in the nucleic-acid containing components of granule and Purkinje cells. Particularly in Purkinje cells, alterations occurred in the cytoplasmic RNA that were secondary effects due to regressive cellular alterations, since swelling of the nerve cells was observed initially and decrease in cytoplasmic nucleic acid content later on. The change in the structural organization of the nuclear DNA in the pyknotic granule cells was possibly also a secondary effect due to the increased density of the pyknotic nuclei. But it could also have represented a primary change in the physiochemical quality of the DNA caused by the action of ionizing radiation.

In the present study, as with the studies mentioned, the granule cells exhibited the morphological changes. Taking all the above points into consideration, the cellular effects observed were considered to be due to ionizing events in the cells.

METHYL MERCURY INSULT

In the present study, after the single dosages of methylmercuric chloride, the granule cells displayed neuronal changes in the form of pyknosis and karyorrhexis while the Purkinje and other cerebellar cells remained unaltered. Two main types of degenerative changes have been observed in the granule cells following organic mercury poisoning.[75] Type I: Coagulative changes--cells became very dark and electron dense, probably as a result of acute coagulative necrosis. Type II: Lucid change--cells had lost their normal chromatin pattern and appeared to electron lucid. Miyakawa and Deshimaru[76] suggested that some nuclear material may have been lost in these cells and that the nuclear envelopes appeared to have a compensatory thickening. These two degenerative changes that were just described probably correspond to the light microscopic observation of dark and light cells described by Takeuchi et al.[77] in the granular layer of the cerebellum. These dark and light cells were also observed in the larger mercury-dosed group but not to any large extent as in the case of the above study.

The differences in methyl mercury uptake observed between various nerve cells could be due to the structural or metabolic properties of certain neurons, which would, therefore, be more susceptible to the toxic action of mercury. In this respect several cell populations with different degrees of susceptibility may exist in a complex anatomical arrangement such as the brain.

In a study using ^{203}Hg, it was demonstrated that the Purkinje cells were more heavily labelled than the granule cells; however, as was found in the present study, the degeneration in the granular layer was much more extensive.[78] This may be due to the small size of the granule cell in comparison with the Purkinje cell. Although the absolute content of mercury in a granule cell was low, the concentration of mercury in a granule cell could be actually higher than that in the Purkinje cell. Furthermore, Kosmider[79] demonstrated histochemically that the Purkinje cells were very rich in sulfhydral groups, and that the histochemical reaction was greatly reduced after mercury poisoning. This may suggest that the large amounts of sulfhydral groups in the Purkinje cells may offer a quencing effect on the mercury action inside the cell, rendering a higher mercury tolerance.

The different responses of the neurons that were shown in this study were probably related to the differences in the cellular metabolism. Likewise the absorption, distribution, elimination, and tolerance of mercury by different cells may vary.[80]

Yoshino et al.[81] suggested that the susceptibility of cells to organic mercury compounds may depend upon their protein synthesizing activity. However, mere rate of protein synthesis cannot be the critical factor since in the cerebellum Purkinje cells were much more active in this respect than granule cells, so this is an area needing much more investigation before it is applied to the results of this study.

CO-INSULT

Both the co-insult groups exhibited fewer cellular alterations than at least one of their counterpart single insults. The lower co-insult group (E) had more of an effect than its counterpart mercury single insult (D); however, the same single dosage of radiation did more cell damage. The larger co-insult treatment group (G) demonstrated less morphological change than either of the larger insults alone.

The observation that the co-insult groups exhibited fewer changes than induced by radiation alone produced two possible hypotheses. Either the two insults had a neutralizing effect on each other or the mercury set up a protective mechanism so as to reduce the effectiveness of the radiation. Results of other studies presented in this report favor the latter hypothesis; however, the former cannot be ruled out. Elucidation of the mechanisms involved must await further investigation.

Whatever the reasons may be for the co-insults producing less damage than the single insults, the study did test the hypothesis that the co-insult would have a more deleterous effect than the single insult, and it was proven to be incorrect.

CONCLUSIONS

The cerebellum proved to be the most susceptible area of the brain to mercury and irradiation because of the sensitivity of some of its cells, especially the granular cells. Why specifically the granular cells of the cerebellum exhibited the greatest sensitivity is not known, but the small size of the granule cell may be a strong determining factor. The size of dose and time lapse must also have a bearing on the degree of damage in these granular cells.

The single insult studies corresponded with the findings in the literature with possibly only subtle differences due to the dosages and time intervals. However, these studies needed to be done to link their findings with those of the co-insult studies.

The hypothesis that co-insult treatments would have a more deterimental effect than single insult treatments was rejected because the co-insults had less of an effect on the cells. Because of this finding more hypotheses were presented: 1) The co-insults have a neutralizing effect on each other so as to make their effects less than if administered singly or 2) The mercury causes certain biochemical changes to set up a protective mechanism against the radiation. According to this study, any of these hypotheses would be feasible.

Future work in co-insult studies is needed before any theory can be formed, and even then it is probable that many factors are involved in each co-insult study. The amount of time between treatments and before sacrifice needs to be varied as well as the dosages. Many combinations of the same type of experiment need to take place before the complete story is told. Since there is no doubt that the cerebellum is affected, more extensive work using electron microscopy should be done to find what is happening at the ultrastructural level.

SECTION VII

RESULTS OBTAINED WHEN METHYL MERCURY WAS ADMINISTERED IMMEDIATELY OR 24 HOURS AFTER HEAD IRRADIATION

A. RAT MORTALITY FOLLOWING ADMINISTRATION OF SINGLE AND CO-INSULTS WITH METHYLMERCURIC CHLORIDE IMMEDIATELY AFTER X-RADIATION

Two experiments, designed to investigate the separate and combined mortality effects of methylmercuric chloride (MMC) and x-radiation upon the albino rat, were performed. The first experiment included 80 three-month-old male rats, having a mean weight of 339.8 g and ranging between 312 g and 378 g. The second experiment included 80 three-month-old female rats, ranging in weight from 226 g to 290 g, and averaging 251.9 g.

In each experiment the 80 rats were divided into four groups, each containing 20 animals. One group served as a control, receiving sham irradiation treatment and an intraperitoneal injection of physiological saline. A second group was irradiated with 10,000 R x-radiation to the head only. A third group received only MMC injected intraperitoneally in distilled water. A fourth group was treated with both insults.

In the first experiment each male rat was given 2.75 mg of MMC dissolved in 1.1 ml of distilled water. The female rats used in the second experiment were given 1 mg of MMC per 100 g of body weight. All MMC was injected intraperitoneally immediately after irradiation or sham-irradiation.

The results of the first experiment are summarized in Table 23. The graphed results suggest the possibility that the mortality resulting from MMC intoxication may be depressed by the effects of x-radiation. The results of the second experiment, involving female rats, are summarized in Table 24. Although the results of this experiment tend to follow the pattern observed in the first experiment, the depression of early (Days 1-6 post treatment) MMC-induced mortality in the co-insult group appears less distinct in the second experiment.

Table 23. ACCUMULATIVE PERCENTAGE MORTALITY IN MALE RATS FOLLOWING TREATMENT WITH SINGLE AND COMBINED INSULTS OF 2.75 mg. METHYL MERCURIC CHLORIDE (MMC) AND 10,000 R X-RADIATION TO THE HEAD

	Group*			
Day	A (Control)	B (MMC)	C(X-ray)	D (Co-insult)
1	0	10	0	20
2	0	20	0	20
3	0	30	0	20
4	0	30	0	20
5	0	30	0	20
6	0	40	0	20
7	0	45	0	25
8	0	50	25	50
9	0	50	90	100
10	0	60	100	100
11	0	60	100	100
12	0	65	100	100
13	0	65	100	100
14	0	65	100	100
15	0	65	100	100
16	0	70	100	100
17	0	70	100	100
18	0	70	100	100
19	0	70	100	100
20	0	70	100	100
21	0	70	100	100
22	0	70	100	100
23	0	70	100	100
24	0	70	100	100
25	0	70	100	100
26	0	70	100	100
27	0	70	100	100
28	0	70	100	100
29	0	70	100	100
30	0	70	100	100

*Each group contained 20 rats

Table 24. ACCUMULATIVE PERCENTAGE MORTALITY IN FEMALE RATS FOLLOWING SINGLE AND COMBINED INSULTS WITH 1.0 mg. METHYL MERCURIC CHLORIDE PER 100 g. OF BODY WEIGHT AND 10,000 R X-RADIATION TO THE HEAD

	Group*			
Day	A (Control)	B (MMC)	C (X-ray)	D (Co-insult)
1	0	30	0	20
2	0	40	0	35
3	0	40	0	40
4	0	60	0	50
5	0	65	0	55
6	0	65	0	60
7	0	70	15	60
8	0	70	40	65
9	0	80	100	90
10	0	85	100	100
11	0	85	100	100
12	0	85	100	100
13	0	85	100	100
14	0	85	100	100
15	0	85	100	100
16	0	90	100	100
17	0	90	100	100
18	0	90	100	100
19	0	90	100	100
20	0	90	100	100
21	0	90	100	100
22	0	90	100	100
23	0	90	100	100
24	0	90	100	100
25	0	90	100	100
26	0	90	100	100
27	0	90	100	100
28	0	90	100	100
29	0	90	100	100
30	0	90	100	100

*Each group contained 20 rats

A possible reason for the less clear-cut effect in the second experiment is that the average dose of MMC per animal was greater than in the first experiment. The animals in the first experiment received approximately 0.8 mg of MMC per 100 g body weight, whereas the rats in the second experiment received 1 mg per 100 g of body weight, thus possibly overloading the protective mechanism elicited by the radiation.

These two experiments suggest that a protective mechanism against MMC intoxication is triggered by the effects of x-irradiation. However, since 10,000 R head irradiation results in one hundred percent death by day 10 post-irradiation, any depression of MMC-induced mortality beyond this period cannot be monitored by this type of experiment.

B. RAT MORTALITY FOLLOWING METHYLMERCURIC CHLORIDE ALONE OR 24 HOURS AFTER X-IRRADIATION

Twenty-eight male rats weighing 324 to 377 g were used in this preliminary study. Fourteen rats were injected intraperitoneally at a dose rate of 7 mg/kg body weight with a solution containing 4 mg methyl mercury per ml of physiological saline and received sham irradiation; however, one received an incomplete mercury dose and was removed from the experiment. Fourteen rats received 10,000 R of x-rays at a dose rate of 265 R/min and methyl mercury as above.

Excess deaths were noted in the co-insult group. Four of 14 irradiated animals died until the third day. One animal in each group died during the third day. Additional sham-irradiated animals died on the 4th and 6th days after irradiation, but no further deaths in the co-insult group were observed until the 7th day. All of the co-insult group were dead by the 10th day, due to the effects of the large dose of radiation.

Further experiments will be required to establish the pattern of deaths which occur after various doses when irradiation precedes the methyl mercury insult. It is probable that excess deaths during the first 24 hours is due to increased blood-brain barrier permeability caused by irradiation insult. Lack of further deaths in the co-insult group could be due to possible protection caused by proliferation of peroxisomes (see subsequent section in this report on this subject), or the more susceptible animals may have died within the first 24 hours following the co-insult, while they survived for a longer period when mercury only was administered. Replication of this experiment with larger numbers of animals and other experiments varying the mercury and radiation doses and the time between insults will be required to more clearly establish the nature of the interaction of the two insults.

SECTION VIII

EFFECTS OF COMBINED INSULTS OF METHYLMERCURIC CHLORIDE AND X-RADIATION UPON THE UPTAKE OF SULFUR-35 BY THE RAT BRAIN*

INTRODUCTION

Studies evaluating the effects of single insults of x-radiation and methyl mercury upon the blood-brain-barrier (BBB) have been done. Nair and Roth[82] demonstrated a BBB against sulfate, noting an increased in vivo uptake of sulfate by the rat 48 hours after 10 kR head x-irradiation. Steinwall and Olsson[83] found that the presence of methyl mercury dicyandiamide, as well as $HgCl_2$, brought about a change in BBB permeability, increasing the influx of the fluorescent Evans blue-protein complex while reducing the penetration of another indicator (Se^{75}-selenomethionine).

Results presented earlier in this report have shown that the effects of the single insults upon brain serotonin and norepinephrine levels tended to be neutralized in the co-insulted animals. We have also observed that large doses of x-irradiation tended to reduce methyl mercury-induced mortality. The present study evaluates the interaction of single and combined doses of x-radiation and methyl mercury upon the in vivo uptake of radioactive sulfate by various brain regions.

METHODS

Ninety 90-day-old male Sprague-Dawley rats, weighing 230 to 324 g, were divided into 9 groups containing 10 animals per group as shown in Fig. 4. Animals were maintained under standard laboratory conditions, housed 4 to a cage, and received food and water ad libitum. Each group received a different dose combination of intraperitoneally injected methyl mercury and head x-irradiation. The methyl mercury doses were either 0,

*A portion of these results were contained in a dissertation submitted by James Earhart to North Texas State University in partial fulfillment of the requirements for the Ph.D. degree. These results were presented by E.W. Hupp, in a paper co-authored by Earhart and Hupp at the Radiation Research Society meeting, June, 1976. The abstract is in press in Radiat. Res.

Figure 4. Experimental design for studying the effects of single and co-insults of methylmercuric chloride and x-radiation upon the uptake of sulfur-35 sodium sulfate by various brain areas

X-Radiation to Head in kR	Methylmercuric Chloride Injected Intraperitoneally (mg/kg of Body Weight)		
	0	4.03	8.06
0	10 animals	10 animals	10 animals
5	10 animals	10 animals	10 animals
10	10 animals	10 animals	10 animals

4.03 or 8.06 mg/kg body weight. The x-ray doses of either 0 kR, 5 kR, or 10 kR were delivered to unanesthetized animals by a General Electric Maximar 250 KVP X-Ray Unit at a target distance of 170 mm and a dose rate of 264.5 R/minute. The filters used were 0.5 mm Cu and 1.0 mm Al. During irradiation the body was shielded by a 5.4 cm thick layer of lead. Sham-irradiated control animals were restrained for the same length of time as animals receiving radiation. All animals were anesthetized with pentobarbitol (40 mg/kg) and killed by immersion in liquid nitrogen 48 hours after irradiation. Five minutes prior to sacrifice, each anesthetized animal was injected in the femoral vein with sulfur-35 sodium sulfate (30 µc per 100 g), based upon the findings of Nair and Roth[84] that this dosage resulted in measurable increases of that indicator in the rat brain at 48 hours post-irradiation. Immediately before sacrifice, 1 ml whole blood samples were taken by heart puncture for liquid scintillation counting.

Tissue samples of caudate nucleus, cerebral cortex, thalamus, hypothalamus, hippocampus, inferior colliculus, medulla, cerebellum, skeletal muscle and liver were taken for liquid scintillation counting and dry weight determination. Frontal sections of the frozen rat brain were sliced at intervals designed to expose each brain area to be sampled. The sections were laid flat on the dissection plate of a dry-ice box and desired samples were carved from the sections with a scalpel. Tissue samples were prepared for counting after Mahlin and Lofberg[85] and counting was done on a Beckman Model LS-200 Scintillation Counter. Radioactivity was determined for each sample and the results were expressed as percent of the amount of radioactivity contained in the blood.[86] The percent of blood concentration values (PBC values) computed for each sample made comparisons of sulfate uptake by various tissues possible. The PBC value for each sample is computed as follows:

$$\text{PBC} = \frac{\text{CPM/mg dry wt}}{\text{CPM/}\mu\text{l whole blood}} \times 10^2$$

RESULTS

Percent of Blood Concentration (PBC) values for each type of sampled tissue are tabulated in Table 25. An analysis of variance was run for the PBC values of each tissue-type. When significant differences between PBC values were indicated, Duncan's Multiple Range Test was performed to determine the groups between which significant differences occurred.

Analysis of the various brain regions revealed several patterns of sulfate uptake following the administration of single and co-insults of methylmercuric chloride and x-radiation. When differences were found to exist between the single and combined insults, the co-insult effects tended to neutralize each other. In some cases, however, one insult tended to override the effect of the other insult. The various patterns of neutralization are summarized below.

Table 25. COMPARISON OF PERCENT OF BLOOD CONCENTRATION (PBC) VALUES OBTAINED BY TREATING RATS WITH VARYING DOSES OF SINGLE AND CO-INSULTS OF METHYLMERCURIC CHLORIDE AND X-IRRADIATION TO THE HEAD

Anatomical Area or Tissue	Control	5 kR	10 kR	4.03mg MMC/kg	8.06mg MMC/kg	5 kR + 4.03mg MMC/kg	5 kR + 8.06mg MMC/kg	10 kR + 4.03mg MMC/kg	10 kR + 8.06mg MMC/kg
Cerebral Cortex	20.8	35.0	30.6	29.7	17.1	30.0	27.6	30.8	29.4
Caudate Nucleus	21.7	27.9	36.7	22.4	19.1	24.7	23.0	26.5	25.5
Thalamus	14.2	25.8	30.0	19.9	17.0	21.2	17.7	19.7	22.3
Hypothalamus	28.5	29.9	39.2	25.1	18.2	25.9	25.7	41.4	33.5
Hippocampus	29.4	28.3	32.2	23.0	20.2	31.6	30.1	31.7	31.0
Inferior Colliculus	32.8	39.0	39.1	35.4	32.2	39.4	32.1	35.7	33.5
Medulla	20.8	23.3	25.3	20.9	23.2	22.5	24.2	26.1	21.7
Cerebellum	42.1	43.3	32.8	39.2	26.3	43.8	36.0	33.2	44.8
Skeletal Muscle	89.6	75.0	54.5	76.2	80.4	55.2	60.4	53.6	50.1
Liver	263.0	259.7	246.0	240.6	182.0	249.4	265.5	218.4	222.0

A summary of the effects of the single insults and co-insult combinations is given in Table 26. Increases or decreases in sulfate uptake by various tissues were categorized according to whether they were statistically significant changes or whether they were not statistically significant changes. The latter were arbitrarily defined as equal to or greater than 10% of control values, although not statistically significant.

SUMMARY OF SINGLE INSULT EFFECTS

As indicated by Table 26, the x-radiation insult has a strong tendency to cause increased uptake of sulfate by the brain. Ten kR x-irradiation, for example, causes uptake in all brain regions monitored except the hippocampus and the cerebellum. The uptake of sulfate by the hippocampus after treatment with 10 kR was the same as that in the controls, while the cerebellum showed only a numerical decrease in uptake. Treatment with 5 kR x-irradiation produced a similar but less pronounced pattern to that obtained with 10 kR x-irradiation.

Methyl mercury tends to have an effect opposite to that of x-irradiation upon the uptake of sulfate by brain tissues, with methyl mercury generally causing a decrease in sulfate uptake. The only statistically significant increase in sulfate uptake was indicated for the cerebral cortex after treatment with the smaller dose of methyl mercury. This is unexplained, especially in view of the fact that the larger dosage causes a numerical decrease in sulfate uptake by the cerebral cortex.

SUMMARY OF CO-INSULT EFFECTS

The pattern most often seen after co-insult treatment is a neutralization of effects. In the hypothalamus after co-insult treatment with 10 kR and 8.06 mg methyl mercury/kg of body weight, a statistically clear case of cancellation of effects is seen. The single insults cause uptake changes in opposite directions: 10 kR x-irradiation resulting in a significant increase in sulfate uptake and the larger dose of methyl mercury resulting in a significantly reduced uptake of sulfate. The co-insult treatment, however, produces no effect which is significantly different from the control value. Similar, although not significant, patterns are seen in the medulla after treatment with the largest co-insult doses and in the caudate nucleus after co-insult treatment with lower radiation and the higher methyl mercury doses.

X-irradiation tends to override the effect of methyl mercury in a number of tissues. A case in point is the hippocampus, in which sulfate uptake is significantly decreased by the larger dose of methyl mercury while the sulfate uptake after the larger co-insult treatment is no different from the control or the 10 kR single insult treatment uptake. Similar patterns may be seen in the cerebellum, hypothalamus and medulla.

Table 26. COMPARISON OF S^{35}-SODIUM SULFATE UPTAKE BY TEN BODY TISSUES IN RATS TREATED WITH SINGLE AND CO-INSULTS OF METHYLMERCURIC CHLORIDE (MMC) AND X-IRRADIATION

Treatment: X-Irradiation (kR) + MMC (mg/kg)	Increased Uptake of S^{35}-Sulfate		Uptake of S^{35}-Sulfate Same as Control	Decreased Uptake of S^{35}-Sulfate	
	Sig.	Not Sig.		Sig.	Not Sig.
10.0 + 0.0	CN, CC, T, HT	I, M	HI, L	S	CE
5.0 + 0.0	CC, T	I, CN	HT, HI, M, CE, L		S
0.0 + 8.06		T, M	I	HT, CE, L, HI	CN, CC, S
0.0 + 4.03	CC	T	CN, CE, I, L, M		HT, HI, S
10.0 + 8.06	T, CC	CN, HT, CE	I, M, HI	S	L
10.0 + 4.03	HT, CC	CN, M, T	HI, I	S	CE, L
5.0 + 4.03	T, CC	CN, I, CE	HI, HE, M, L	S	

Abbreviations: CN - caudate nucleus, CE - cerebellum, CC-cerebral cortex, HI - hippocampus, HT - hypothalamus, I - inferior colliculus, M - medulla, T - thalamus, L - liver, and S - skeletal muscle. Sig. differences are significant at $p<0.05$ (Duncan's Range Test). Not sig. differences represent changes $\geq$ 10% of control values, although not statistically significant.

In other tissues methyl mercury tends to override the effect of x-irradiation. A treatment of rat heads with 10 kR causes a significant increase in sulfate uptake by the caudate nucleus, while the larger dose of methyl mercury causes a numerical decrease in uptake not significantly different from the control value. The larger co-insult causes a numerical increase in sulfate uptake that does not differ significantly from the control value. Similar observations are made concerning the thalamus, inferior colliculus and cerebellum.

DISCUSSION

Insults of both x-irradiation and methyl mercury demonstrate the presence of a BBB acting against radioactive sulfate, with irradiation disrupting and methyl mercury enhancing the barrier effect. Our results with the 10 kR single insult corroborate the work of Nair and Roth,[87] in which they demonstrated that increased brain-uptake of sulfate occurs following 10 kR head x-irradiation.

The current study demonstrated that the BBB to sulfate may be enhanced by the intraperitoneal administration of methyl mercury. This finding is consistent with the observations of Steinwall and Olsson[88] that methyl mercury dicyandiamide given at a dose rate of 200 mg/kg body weight resulted in a decreased uptake of ^{75}Se-selenomethionine by the brain. That the BBB is complex is indicated by the fact that these investigators also found an increased permeability of the cerebral blood vessels to Evans blue dye. They obtained the same results with ^{75}Se-selenomethionine and Evans blue after treatment with the inorganic mercuric chloride. This suggests that the greater toxicity of organic mercurials may be a result of phenomena other than BBB damage.

Although the mechanism for transporting sulfate across the BBB is not known, there are indications that in a number of both procaryotic and eucaryotic cells sulfate-uptake is accomplished by active transport.[89] Since, in general, mercurials are powerful but unspecific enzyme inhibitors,[90] it seems likely that reduced sulfate-uptake in the rat brain results from methyl mercury poisoning of an active transport enzyme system.

The specific locality of BBB lesions caused by x-irradiation is not known. However, if the capillary endothelium with its tight intercellular junctions is the major anatomical component of the BBB,[91] then it is likely that the radiation-induced lesion occurs somewhere in that structure. Maximum disruption has been found to occur at 48 hours after irradiation,[92] the delayed action suggesting that products such as lipoperoxides resulting from the ionization activities of radiation are involved in causing the barrier lesions. Possible damage is done to either the intercellular tight junction or to some other component of the cell membrane. Evidence for in vitro radiation damage to cancer cell membranes through the vehicle of H_2O_2 has been reported.[93]

Whatever the operative mechanisms involved in the BBB-altering abilities of x-irradiation and methyl mercury, they tend to neutralize or counteract each other when administered as co-insults. This suggests that they are attacking different transport pathways. It seems possible that x-radiation may be causing anatomical leaks, while methyl mercury is inhibiting an enzyme system mediating the transport of sulfate.

SUMMARY

This study was designed to investigate the interaction of methyl mercury and 10 kR x-irradiation relative to the uptake of the indicator ^{35}S-sodium sulfate by various regions of the brain. Several general trends emerged. First, as previously reported in the literature, acute doses of x-irradiation tended to cause an increase in uptake of sulfate by the brain areas examined. Second, methyl mercury was found to have an opposite effect upon the passage of sulfate across the BBB, decreasing its rate of passage. Third, the general interaction pattern produced by the co-insult was neutralization. In a few tissues a net cancellation of effects was seen. However, more often an overriding of effects upon the brain appears to be a typical pattern for the co-insult with methyl mercury and x-irradiation.

SECTION IX

PEROXIDE INDUCED PROTECTION AGAINST METHYLMERCURIC CHLORIDE TOXICITY*

INTRODUCTION

Since in the results reported earlier 10 kR x-irradiation to the head causes an increased permeability of the rat blood-brain barrier (BBB) to S^{35}-sodium sulfate and to the anticonvulsant drug acetazolamide,[95] we proposed that a co-insult with methyl mercury and x-irradiation might result in a more rapid uptake of methyl mercury and thus earlier death. However, during the period prior to total killing by x-irradiation we observed up to 20% lower mortality for co-insulted rats than for rats receiving only methyl mercury. The lowered mortality rate observed in the co-insulted animals was opposite to our expectations, and suggested that x-irradiation protects against methyl mercury. Since 10 kR to the head caused death between days 8 and 10 after irradiation, the period for evaluating the protective effect was limited.

A less severe insult, mimicking the x-irradiation effect, that allowed the animals to live long enough for further investigation of the protective mechanism was needed. Lipid peroxidation occurs in irradiated tissue homogenates[96] and lipoperoxides accumulate in rat liver following 1.5 kR whole body x-irradiation.[97] There is in vivo evidence that radiation-induced peroxidation is responsible for cell damage,[98] while in vitro experiments demonstrate that radiation-produced hydrogen peroxide can increase permeability of murine lymphoma cells.[99] Based upon the observation that x-irradiation protects against methyl mercury toxicity and upon evidence from the literature that radiation-induced peroxides are responsible for the secondary effects of ionizing radiation, we chose hydrogen peroxide (HP) for use in a pretreatment regime designed to simulate radiation effects and to trigger the proposed protective mechanism. Several experiments were conducted, each based upon the results obtained in the previous experiment.

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*Prepared from a manuscript to be submitted for publication. Authorship: Earhart and Hupp

PROCEDURE

Sprague-Dawley and Sprague-Dawley-derived rats, used in these studies, were housed in wire-mesh cages under controlled conditions of lighting (day-night cycle of 13-11 hrs) and temperature (22°C). Each animal was fed standard laboratory chow and water ad libitum.

For the first study one hundred 90-day-old male and female rats were divided into two groups of 50 each: one group received a pretreatment with five 1 ml doses of 1.5% HP injected intraperitoneally at 24 hour intervals and the other group received sham pretreatment with physiosaline. Forty-eight hours after the last injection of either peroxide or saline, each animal was given 10 mg methyl mercury per kg of rat. The animals were observed for 30 days, and deaths recorded to the nearest day.

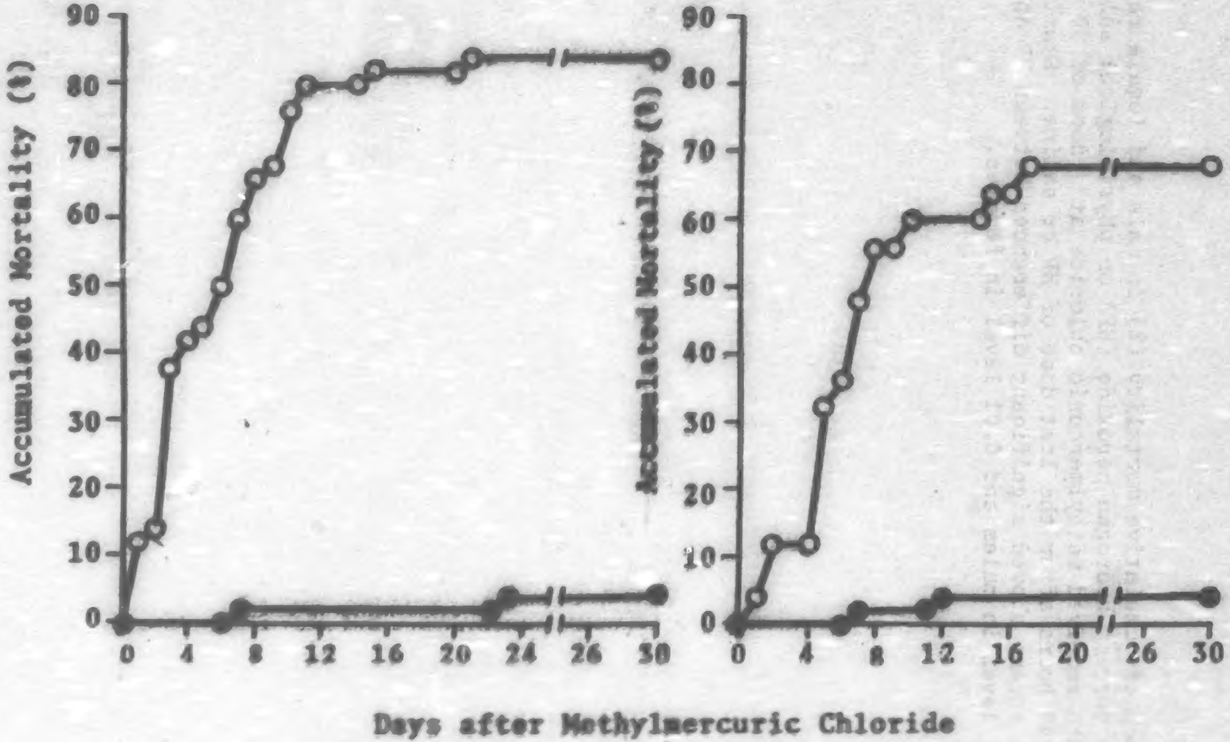
In the second experiment, ninety 189 to 249 g female rats were randomly divided into groups of 30 animals each. Half the rats in each group were subjected to the hydrogen peroxide pretreatment regime, while the other half received sham pretreatment with saline. The three groups were intraperitoneally injected with methyl mercury at doses of 10.0, 12.5 and 15.0 mg/kg. The rats were observed for 30 days, during which time deaths were tabulated.

In the third experiment, 217 to 283 g female rats were randomly divided into 2 groups of 5 animals each. One group received HP pretreatment, while the other groups received sham pretreatment with saline. Animals were killed by decapitation 48 hours after the last dose of HP or saline. Brains were removed and sections of the nucleus arcuatus prepared after the technique of Srebro.[100] Peroxisome-like organelle system-containing glial cells were counted in 0.01337 mm^3 of the nucleus arcuatus of each rat brain.

RESULTS

The results obtained in the first experiment (Fig. 5) demonstrate that the HP pretreatment provided significant ($P < 0.01$) protection against methyl mercury-induced mortality. Eighty-four percent of the control males and 68% of the control females died compared to 4% and 8%, respectively, of the protected animals.

Figure 5. Comparison of cumulative mortality (%) in male and female rats pretreated with 1.5 percent hydrogen peroxide (HP) or physiological saline for 5 days. The rats received methylmercuric chloride at a dose of 10 mg per kg body weight 48 hours after the last dose of HP or saline. Statistical analysis by Chi square showed significant differences between HP and saline at the 0.001 level in males and 0.01 level in females.


Accumulated Mortality (%)
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
26
30
Accumulated Mortality (%)
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
26
30
Days after Methylmercuric Chloride

Although the manner in which the peroxide-induced protective mechanism (PIPM) operates is not understood, several observations suggest a hypothesis. It is unlikely that any type of direct chemical relationship exists between HP and methyl mercury. Since methyl mercury was administered 48 hours after the last dose of peroxide, the catalase activity probably had inactivated the injected HP. If a methyl mercury-HP complex is responsible for the PIPM, one might expect that some protection would be achieved with a HP post-treatment regime. However, when we began injecting methyl mercury (10 mg/kg), no reduction in mortality was observed. Consequently, the proposed PLOS, demonstrated in the perivascular glia of the periventricular regions of the brain[101] might be a possible response mechanism for protecting against methyl mercury toxicity.

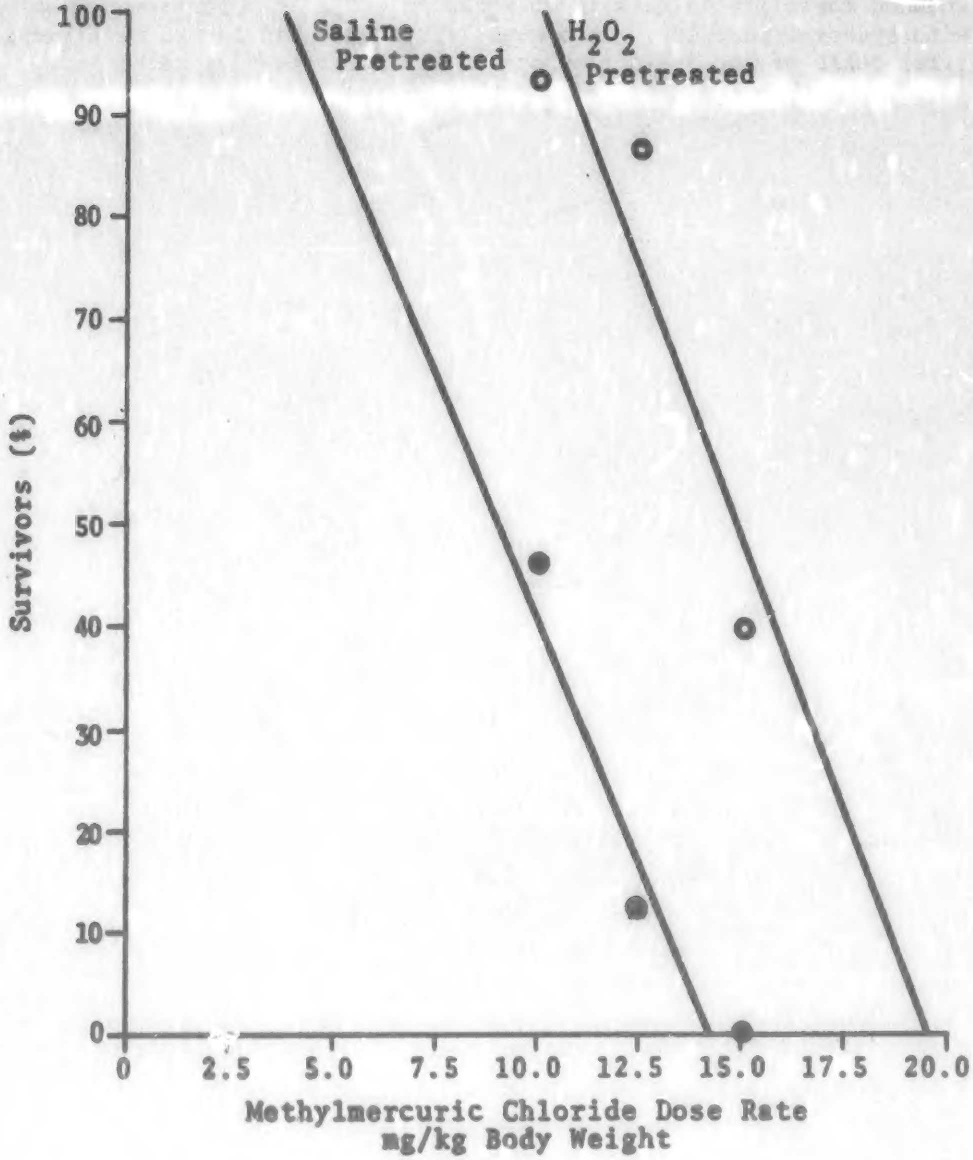
Because of its high concentration of sulfhydryl groups, its perivascular location and its proliferative response to ionizing radiation, the PLOS appears to be suited for an important role in protection against heavy metals. Several studies lend support to such a protective phenomenon.[102]

We suspected that the PLOS could offer protection only to the extent of its level of proliferation; hydrogen peroxide or saline pretreated rats were therefore subjected to graded doses of methyl mercury. The percentages of rats surviving the various doses of methyl mercury are shown graphically in Fig. 6.

At the 10 mg/kg dose rate, for example, 93% of the HP pretreated rats survived compared to 47% of the saline pretreated rats. This protective activity of the HP pretreatment regime is apparent at each dose level. Also, in both HP and saline pretreated groups the percentage of survivors decreased with increasing doses of methyl mercury. A regression line analysis of the survival data suggests that a methyl mercury dosage of about 19.4 mg/kg is sufficient to overcome the protective effect of the HP pretreatment compared to the 14.4 mg/kg required to kill all untreated rats. This represents a 36% increase in protection.

In order to evaluate PLOS proliferation, a small-scale study was conducted in which the number of PLOS containing glial cells in sections of the nucleus arcuatus were counted. Means of 139.4 and 183.4 were obtained for saline and HP pretreated rats, respectively. This represents a 32% increase in PLOS-containing glial cells following HP pretreatment, which compares favorably with the 36% increase in protection provided by the treatment. This supports the hypothesis that proliferation of the PLOS is responsible for, or an indication of mechanisms responsible for, the protection provided by HP against methyl mercury-induced lethality. Further studies of other tissues will be necessary to substantiate the hypothesis.

Figure 6. A regression line analysis of the survival response to graded doses of methylmercuric chloride exhibited by hydrogen peroxide pretreated (open circles) and saline pretreated (closed circles) 90-day-old female rats.


Saline
Pretreated
H_2O_2
Pretreated
Survivors (%)
100
90
80
70
60
50
40
30
20
10
0
0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Methylmercuric Chloride Dose Rate
mg/kg Body Weight

SUMMARY

A highly significant level of protection against methylmercuric chloride-induced mortality in rats is observed following a pretreatment regime with hydrogen peroxide. An organelle system found in the perivascular glial cells of the brain is proposed as the protective mechanism.

CO-INSULTS EFFECTS OF METHYL MERCURY AND RADIATION WITH DIFFERENT TEMPORAL RELATIONSHIPS*

INTRODUCTION

In our earlier studies of co-insult effects of radiation and methyl mercury, we evaluated the animals for only a few hours after the second insult and then killed them for histological and biochemical studies and for mercury analysis. Long-term analysis was not possible because the radiation dose was lethal in 2-4 days following total body irradiation, or in 8-10 days following head-only irradiation, due to damage to the gastrointestinal system. In those studies, where two insults were used, methyl mercury was administered 7 days before the radiation, or 7 days before testing if used alone, to allow time for the mercury to localize in sensitive tissues and produce an effect.

PROCEDURE

In the study to be reported here, the dose of each insult which was lethal to approximately 50% of the animals was determined; then 100% of this dose, 75% of this dose, and 50% of this dose was administered, either separately or together. Various time relationships were used for each level of insult. These relationships will be described in Table 28, which contains all the groups. Because of limitations on the number of animals which could be housed, treated and tested, the study was conducted in three phases. Each phase contained all time relationships but only one dose level, i.e., either 100%, 75% or 50% of the $LD_{50/30}$. There were two runs within each phase; after comparison between runs, data was combined for runs within phases. Because of the design, most comparisons will be limited to comparisons between treatments within level of dose.

The rats were tested for behavior using an open-field observation area previously described. Briefly, the area consisted of a circular open field 81 cm in diameter surrounded by a 33 cm high wall. The field was divided by two concentric circles and lines radiating from the center into 12 truncated triangular-shaped areas surrounding a central circular area. The open field test consisted of placing the subject in the central circular area of the field and observing the subject's activity for a period of 2 minutes. The following measures of emotionality and ambulation were recorded: (a) the number of defecations, (b) the number of areas entered with all four feet (ambulation), and (c) the number of times the animal stood on its hind legs (rearings). The animals were tested two hours after the second insult, then 2, 4, and 7 days later, then twice weekly until 31 days after the second insult. Data collection in all tests was conducted under a blind procedure.

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*Presented at Texas Academy of Sciences, March, 1976, Nancy Partlow and E. W. Hupp, authors.

RESULTS

LETHALITY

Data from which the $LD_{50/30}$ for methylmercuric chloride could be determintd had been determined earlier; we did, however, have to determine the $LD_{50/30}$ for gamma radiation, using our dosimetry and for our strain of rat. The results are presented in Table 27. Since 40% of the rats survived at doses of 725, 750 and 775 R, the dose of 750 R was selected as the $LD_{50/30}$ radiation dose.

The lethality observed in the various treatment groups is presented in detail in Table 28 and summarized in Table 29. In the earlier studies using large radiation doses, an apparent antagonism existed between the two insults in the first few hours after the second insult, making the co-insult less effective than either treatment alone. In contrast, the results obtained in this study with lower doses and longer time periods show the effects of the two insults to be partially additive. Results for single insults were as expected, the calculated $LD_{50/30}$ yielding 40% and 70% lethality, well within the expected range for a sample size of 10. However, 98% of the animals receiving the $LD_{50/30}$ of both insults died. The groups receiving 75% of the $LD_{50/30}$ of a single insult exhibited 52% lethality, closely approximating an LD_{50}. Thus the effects of the two insults are partially additive: i.e., if fully additive, 50% of the $LD_{50/30}$ of each insult would have resulted in 50% lethality where only 4% was observed, while no additivity should have shown the same effect in co-insult groups as in single insult groups. The results presented in Table 28 indicate that within the time intervals used in this study, the temporal relationship between doses did not affect the mortality, so that in all cases co-insults appeared to be equally effective.

BEHAVIOR

Ambulation scores 2 hours after the second insult are presented in Table 30. In the groups receiving 100% of the $LD_{50/30}$, mercury alone or radiation alone produced some reduction in ambulation. In co-insulted

Table 27. PRELIMINARY RADIATION LETHALITY DETERMINATION

Dose in R	Number of Rats	% Survival at 30 days
900	10	0
800	20	5
775	10	40
750	10	40
725	10	40
700	20	60

Table 28. PERCENT LETHALITY AT DIFFERENT LEVELS OF INSULT AND DIFFERENT TIME INTERVALS BETWEEN INSULTS

Treatment*	% of $LD_{50/30}$ 100	75	50
R-7d-Hg	100	70	0
Hg-7d-R	100	50	20
Hg-4d-R	100	60	10
R-4d-Hg	90	50	0
R-1d-Hg	90	40	0
Hg-1d-R	100	40	0
Hg-15m-R	100	60	0
R-15m-Hg	100	50	0
Hg only	40	0	0
R only	70	0	0
Control	0	0	0

*R refers to radiation, the second value the interval in days (d) or minutes (m) between insults, Hg refers to mercury treatment. This format is used in all succeeding tables.

Table 29. SUMMARY OF PERCENT LETHALITY FOR THE CO-INSULT AND THE SINGLE INSULT GROUPS

Treatment	% of $LD_{50/30}$ 100	75	50
All co-insult	98	52	4
Hg only	40	0	0
Rad only	70	0	0
Control	0	0	0

Table 30. AMBULATION OF RATS 2 HOURS AFTER THE SECOND INSULT

Treatment	% of $LD_{50/30}$		
	100	75	50
R-7d-Hg	4.4	10.0	12.2
Hg-7d-R	10.5	18.0	16.7
Hg-4d-R	16.4	25.0	22.0
R-4d-Hg	11.8	10.0	10.5
R-1d-Hg	11.3	7.7	10.7
Hg-1d-R	20.5	21.0	18.7
Hg-15m-R	17.6	16.3	12.9
R-15m-Hg	9.5	10.4	11.4
Hg only	17.9	8.5	15.5
R only	16.7	22.7	17.5
Control	21.6	28.2	18.7

animals, when radiation was the second insult, ambulation was similar to that in the group receiving radiation alone; when mercury was the second insult, the depression was much greater than either insult produced alone. This relationship was observed even in the group where only 15 minutes intervened between insults. A similar pattern was observed in the animals receiving 75% or 50% of the LD_{50}, except at the 50% level of radiation alone or radiation as the second insult, where ambulation did not decrease except in the case of the 15 minute interval between insults. The results show this measure of behavior to be more sensitive in detecting effects of the insults than lethality data. This measure also differentiated between the two insults with regard to the severity of the defect in the lowest dose group, and showed that the temporal relationship between the insults affected the results obtained.

Data on the number of rearings in the tests conducted 2 hours after the second insult is presented in Table 31. The effect on the number of rearings generally agrees with the ambulation data, although in most cases the differences are less marked. Again, 50% or 75% of the $LD_{50/30}$ was nearly as effective as 100% of the $LD_{50/30}$.

The ambulation scores and the number of rearings in tests conducted 2-7 days after the second insult are presented in Tables 32 and 33. All of the animals in the 100% of the LD_{50} group irradiated 7 days before mercury administration died in the period 2-7 days after the second insult, and provided insufficient data to tabulate. The effects on ambulation observed in this time period in the animals receiving 100% of the $LD_{50/30}$ were very similar to those observed 2 hours after the second insult. In the lower dose groups, effects were less pronounced. In the 75% of $LD_{50/30}$ groups radiation alone had a slight effect with a very similar, somewhat lower, activity occurring in the mercury alone and co-insult groups. The depression in activity was less than that observed at 2 hours. It should be recalled that these groups showing similar activity during this time period later exhibited differences in mortality, with 52% of the co-insulted animals dying while none of the mercury only treated animals died. The ambulation scores for the animals receiving 50% of the $LD_{50/30}$ doses did not differ significantly from the control scores.

As was the case 2 hours after treatment, effects on rearings were similar to but less marked than effects on ambulation. Only the groups receiving 100% of the two insults 7 or 4 days apart exhibited a significantly decreased number of rearings during this time period.

The ambulation scores and the number of rearings for the entire 31-day test period are presented in Tables 34 and 35. Since nearly all the co-insulted animals receiving 100% of the $LD_{50/30}$ and 52% of the co-insulted animals died during the test period, removal of animals from the test groups by death may have influenced group means. In most cases, the 31-day mean ambulation score for the animals receiving 100% of the $LD_{50/30}$ was very similar to that observed 2 hours after the second insult.

Table 31. NUMBER OF REARINGS 2 HOURS AFTER THE SECOND INSULT

Treatment	% of $LD_{50/30}$		
	100	75	50
R-7d-Hg	2.4	3.7	9.5
Hg-7d-R	3.1	7.9	7.6
Hg-4d-R	2.8	8.3	9.6
R-4d-Hg	3.1	2.7	2.3
R-1d-Hg	3.8	5.5	5.5
Hg-1d-R	5.2	7.0	8.1
Hg-15m-R	3.5	3.7	5.7
R-15m-Hg	1.9	7.3	4.1
Hg only	3.1	4.5	7.3
R only	3.5	8.4	6.0
Control	4.0	9.1	10.6

Table 32. AMBULATION OF RATS DAYS 2-7

AFTER THE SECOND INSULT

Treatment	% of $LD_{50/30}$ 100	75	50
R-7d-Hg	----	21.4	20.7
Hg-7d-R	9.0	22.1	17.3
Hg-4d-R	13.2	23.6	17.4
R-4d-Hg	10.5	21.2	19.0
R-1d-Hg	24.2	19.3	19.6
Hg-1d-R	14.5	22.2	21.6
Hg-15m-R	15.9	18.1	23.0
R-15m-Hg	12.9	19.5	19.7
Hg only	19.7	22.6	21.8
R only	14.7	26.9	22.1
Control	25.0	29.9	19.1

Table 33. NUMBER OF REARINGS DAYS 2-7 AFTER THE SECOND INSULT

Treatment	% of $LD_{50/30}$		
	100	75	50
R-7d-Hg	----	6.5	9.1
Hg-7d-R	2.0	6.6	6.5
Hg-4d-R	1.3	5.8	7.0
R-4d-Hg	1.4	5.4	4.9
R-1d-Hg	2.8	5.5	6.7
Hg-1d-R	2.0	5.4	6.8
Hg-15m-R	5.4	6.3	8.5
R-15m-Hg	3.3	9.0	11.2
Hg only	3.7	4.9	7.0
R only	2.5	6.8	6.1
Control	2.9	6.1	5.9

Table 34. MEAN AMBULATION SCORES FOR THE 30 DAY TEST PERIOD

Treatment	% of $LD_{50/30}$ 100	75	50
R-7d-Hg	4.4*	24.0	25.6
Hg-7d-R	9.8*	26.9	25.4
Hg-4d-R	14.0*	28.9	22.5
R-4d-Hg	14.5	23.4	24.1
R-1d-Hg	20.8	27.0	24.4
Hg-1d-R	16.0*	26.5	27.0
Hg-15m-R	16.3*	21.9	23.6
R-15m-Hg	12.1*	24.9	25.4
Hg only	21.5	29.0	28.2
R only	17.2	31.2	24.0
Control	25.5	34.3	22.9

*Data on 1 week or less

Table 35. MEAN NUMBER OF REARINGS

FOR THE 30 DAY TEST PERIOD

Treatment	% of $LD_{50/30}$		
	100	75	50
R-7d-Hg	2.4*	6.2	10.4
Hg-7d-R	2.6*	12.7	11.7
Hg-4d-R	1.7*	10.4	11.9
R-4d-Hg	3.4	8.3	8.1
R-1d-Hg	6.4	8.1	9.1
Hg-1d-R	2.8*	9.5	9.3
Hg-15m-R	4.9*	8.7	10.6
R-15m-Hg	2.9*	12.1	9.6
Hg only	4.2	10.6	9.7
R only	3.7	9.8	8.2
Control	4.1	12.0	8.9

*Based on 7 days or less

Due to recovery with time after treatment, the 31-day mean for the animals receiving 75% of the $LD_{50/30}$ exhibited less effect of the treatment than in the earlier time periods previously discussed. Ambulation in the groups exposed to 50% of the $LD_{50/30}$ did not differ significantly from that of the controls.

Results on the number of rearings for the entire 31-day period are more difficult to interpret. Results in the 100% and 50% groups are similar to those on ambulation, although somewhat more variable. The 75% group exhibit more variability and an apparently greater depression in some co-insult groups. However, a careful examination of the data indicated the apparent difference was due to an increase in the number of rearings as the experiment progressed (probably due to greater familiarity with the testing apparatus) while some groups did not exhibit this difference due to death of 50% or more of the animals in the group. Thus data on the number of rearings appears to be less reliable and less useful than the ambulation data.

CONCLUSIONS

The two insults tested in this study are partially additive in that 100% of the LD_{50} of the two insults applied separately resulted in 98% lethality and 75% of the $LD_{50/30}$ resulted in 52% lethality. Behavioral tests, especially ambulation scores during the first 7 days after the second insult, are more sensitive indicators of damage than lethality data. The behavioral observations were also able to discriminate between groups with regard to which insult was applied first while lethality results did not. However, the ambulation scores of the co-insulted groups exhibiting approximately 50% lethality following 75% of the $LD_{50/30}$ for a single insult were similar to the groups receiving mercury only, in which no deaths occurred.

SECTION XI

BEHAVIORAL OBSERVATIONS IN SQUIRREL MONKEYS (SAIMIRI SCIUREUS) FOLLOWING METHYL MERCURY ADMINISTRATION

INTRODUCTION

A limited number of studies were conducted on squirrel monkeys (Saimiri sciureus) in order to compare the results obtained in rats with those obtained in an experimental primate. The lethality results obtained in preliminary experiments have been presented earlier, as that seemed the most appropriate location for those data. This section contains the results of several behavioral studies. The first series involved methyl mercury as a single insult, since data on this insult alone on squirrel monkeys was not available at the time the study was initiated. The final study involved the co-insult of the two agents under investigation.

Subtle distrubances of behavior appearing before any clinical symptoms have been attributed to methyl mercury poisoning.[102] In order to quantitate such variable parameters, measurements of the conditioned response have been utilized. These behavioral tests serve not only to indicate disorders in the brain, but also to give insight to the state of the whole organism.

Although fetal and newborn animals show more susceptibility to mercury exposure,[103] reports of variable degrees of intellectual and emotional disturbance[104] indicate serious effects in the adult. Due to conflicting published conclusions as to whether mercury exposure affects only motor and visual skills while leaving intellectual skills undamaged[105] the study was designed to observe behavioral changes in Saimiri sciureus resulting from exposure to methylmercuric chloride. General behavior observations were executed in order to find subtle behavioral changes after exposure to methyl mercury. Behavior was quantitatively measured in a Wisconson General Test Apparatus (WGTA) in order to test the hypothesis that changes in conditioned responses occur before any overt pathological effects are perceptible. Electrophysiological measurements were made to investigate possible electrically measurable differences between sighted and blind animals.

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*From a manuscript by Francine Joiner and E. W. Hupp, submitted to Environmental Research.

SUBJECTS

The subjects were four female and 12 male squirrel monkeys. The four females (Group I) were jungle born and of an undetermined, advanced age of approximately 10 years. Four of the males (Group II) were born in the colony at Texas Woman's University and the remaining eight (Group III) were young adult males, jungle born.

INJECTION PROCEDURE

Females were injected intraperitoneally three times over a period of 27 months with 1 ml saline or 4.0, 3.0, 2.0 mg/animal of methylmercuric chloride. The intervals between doses were 17 and 10 months; each animal received the same dosage each time. Males were injected once with 1 ml saline or 6.0, 4.5, or 3.0 mg/kg methylmercuric chloride. The four colony-born males (Group II) were studied a year before experimentation with the eight jungle born males (Group III) began.

BEHAVIORAL OBSERVATIONS

GENERAL. A tape recorder counting off 60 15-second intervals was used to mark the time period in which each behavioral action of the animals was recorded.

WGTA. Males were trained to a criterion of 20 correct responses out of 25 in a modified WGTA. Animals learned to recognize four pairs of visual discriminanda differing in shape and color for a raisin reward. Preinjection and postinjection data consisting of three parameters, latency, duration, and correctness of response were measured from 15 trials per day per animal.

FOOD CONSUMPTION

All pre-feeding food weights and post-feeding weights of remains were recorded in order to estimate daily intake of food. These data were recorded for the females and the four colony-born males. An 18-hour food deprivation schedule was enforced during training and testing of male animals in the WGTA.

ELECTROPHYSIOLOGY

Electroencephalograms (EEG) and electroretinograms (ERG) were obtained with an E & M physiograph. Transistorized amplifiers (type 7070 channel amplifier and type 7171 high-gain coupler) were used with the high frequency filter set at 30 Hz for both EEG and ERG. The time constant was 0.3 for EEG and 3.2 for ERG. Animals were injected with Sodium Pentobarbitol of doses sufficient to produce an anesthesia level obliterating voluntary muscular movement. During a 15 minute period of dark adaptation, drops of a mydriatic, epinephrine, and a topical optic anesthetic were applied to the right eye of each animal. After attaining topical anesthesia and pupil dilation, the ERG electrode was applied. The electrode was a modified version of that used by Ogden and Van Dyk[106] for ERG recording in human infants and young children. The electrode was constructed from a filament of platinum wire with the terminal end bent in circular fashion to form a smooth ending. A saline-soaked corneal wick was fashioned over the electrode. The electrode was gently inserted under the eyelid and the eyelid was then taped closed. Photic stimulation was achieved by manual manipulation of an opaque screen to reveal brief intervals of light from a 6 volt lamp. A reference needle electrode was placed subcutaneously over the superciliary arch. EEG needle electrodes were inserted subcutaneously to record from the temporal area to the medial occipital region. Electrocardiogram (ECG) was also monitored.

RESULTS

BEHAVIORAL OBSERVATIONS

GENERAL. An Average Daily Activity Level consisting of a daily average of all behavioral patterns requiring movement was calculated for Groups I and II. The females (Group I) showed decreases in activity (Table 36) paralleling signs of mercury poisoning but not approaching the severity of decrease in activity of the affected males (Table 37). The extent of decrease in activity for the two high-dose males was directly applicable to the amount of mercury per kg of body weight received by each animal with the level of activity decreasing the most with the highest dosage animal.

WGTA. Of the Group II animals, animal #1 (6.0 mg/kg) and animal #2 (4.5 mg/kg) showed the only changes in WGTA data. Their changes, coinciding with clinical signs of poisoning, showed decreases in correct responses and increases in both latency and duration. Results from Group III concurred with decreases in correct responses in latency and duration for animals 1A (6.0 mg/kg) and 3B (4.5 mg/kg). WGTA changes occurred only in conjunction with clinical signs of illness.

FOOD CONSUMPTION

Females showed no continuing change in food consumption until the decrease for the high-dose animal after the third injection (Table 38). Two males (6.0 mg/kg and 4.5 mg/kg) had significant decreases in food consumption after a single injection (Table 39).

SIGNS OF MERCURY POISONING

Vomiting and temporary anorexia were observed in most of the treated animals immediately after injection. Although both males and females showed apathy, weakness, and fatigue, females showed continuing attention to stimuli even to the day of death. Males showed severe lack of

Table 36. AVERAGE DAILY ACTIVITY LEVEL FOR FIVE 30 DAY PERIODS - GROUP I: FEMALES

An. #	mg/kg	Run 1[a]	Run 2[b]	Run 3[c]	Run 4[d]	Run 5[e]
1	6.5	205.8	177.9	152.1	140.8	138.1
2	4.8	155.9	188.2	210.7	139.0	135.0
3	3.6	205.6	196.8	204.8	172.6	166.5
4	0.0	206.4	172.1	217.6	166.3	159.1

[a] 16 months after first dose, immediately before second dose
[b] immediately after second dose
[c] 4 months after second dose
[d] 9 months after second dose, immediately before third dose
[e] immediately after third dose

Table 37. AVERAGE DAILY ACTIVITY LEVEL
FOR TWO 30 DAY PERIODS - GROUP II: MALES

Animal #	mg/kg	Preinjection	Postinjection
1	6.0	231.5	92.0
2	4.5	246.5	130.2
3	3.0	298.7	279.5
4	0.0	239.3	201.0

Table 38. AVERAGE FOOD CONSUMPTION PER DAY IN GRAMS: GROUP I, FEMALES

Animal #	mg/kg	Run 1[a]	Run 2[b]	Run 3[c]	Run 4[d]
1	6.4	54.8	55.7	56.4	20.8
2	5.1	37.2	31.9	49.0	38.6
3	3.8	40.1	35.5	35.5	50.9
4	0.0	44.7	34.6	48.6	51.1

[a] 16 months after first dose, immediately before second dose

[b] immediately after second dose

[c] 4 months after second dose

[d] immediately after third dose

Table 39. AVERAGE FOOD CONSUMPTION PER DAY IN GRAMS: GROUP II, MALES

Animal #	mg/kg	Preinjection Run	Postinjection Run
1	6.0	86.2	23.2
2	4.5	54.8	29.4
3	3.0	85.0	81.7
4	0.0	66.2	63.0

interest progressing to coma. Two males in Group II became completely blind; one survived and one died. In Group III, both males from the 6.0 mg/kg treatment group became blind before death.

Other signs observed were intention tremors, fine and gross motor incoordination, ataxia, spasticity, paralysis, frequent licking of extremities, and fits of anger. Bleeding from the sutures of the skull was observed in many animals during necropsy.

ELECTROPHYSIOLOGY

All blind animals as well as sighted animals had well defined ERG responses to photic stimulation. Simultaneous changes in EEG were observed for all animals except the blind ones, although responses varied in intensity. Severe tremoring of ill animals were evidenced in the electric recordings even in deep anethesia.

DISCUSSION

Although two animals per treatment group in Group III allowed for statistical measurement, the behavioral measurements did not lend to reliable statistical deduction. The variance between subjects in the same treatment group was so great that any combination of the data cancelled out the effects observed. Evidently there were individual differences in response to methyl mercury poisoning or the response was modified by some unnoted factor. Significant change in the indices measured correlated with the clinically observed symptoms of methyl mercury poisoning. If the animals did not show symptoms of sickness, changes in the variables of the experiment were not observed. The measurements of general activity and food consumption served as competent measures of illness in the animals. Loss of appetite and diminished activity indicated onset of illness.

The electrophysiological investigation showed that blindness was not attributable to impaired retinal function. The corresponding measures of EEG stimulation gave an interesting research direction to follow. Direct recording from visual cortex would serve future investigation.

Although the data for ill animals showed changes in responses in the WGTA, there was no sufficient evidence for decreased memory function except in the state of severe illness. Failure to perform in this case may have been due to the deteriorated general condition of the subject, rather than due to failure to remember the task. Therefore, it was concluded that a exposure to methyl mercury does not permanently alter learning performances in adult Squirrel monkeys.

SECTION XII

COINSULT EXPERIMENT--FEMALE SQUIRREL MONKEYS

INTRODUCTION

This study was conducted to extend the preceding experiments involving mercury only to an experiment involving mercury and radiation as co-insults. The study was also designed to extend previous studies which we have conducted with rats to one using squirrel monkeys. A limited number of studies have been reported for either insult used alone, but to our knowledge, no coinsult studies with monkeys have been done.

PROCEDURE

BEHAVIORAL OBSERVATIONS

The monkeys were subjected to the same training and testing regime in the WGTA as described for the preceding studies. A total of 12 monkeys began initial training, and 11 reached the criteria of 20 correct responses out of 25. Following training, the monkeys were tested for 8 days pre-treatment and 30 days post-treatment.

TREATMENT PROCEDURE

Whole-body radiation was applied with a General Electric X-ray unit operated at 250 KVp, 15 ma, 0.5 mm cu, 1.0 mm Al filtration at a dose rate of 55 R/min. Methylmercuric chloride in physiological saline (3 mg/ml) was injected intraperitoneally. In the coinsult groups, methylmercury was administered within 10 minutes of the end of irradiation. Each group except Group 6 contained two monkeys. Ideally, all blocks in Table 40 should have been filled; however, the number of animals and amount of time available for testing limited the experiment to the groups shown. These groups we selected as those expected to yield the greatest amount of information.

Table 40. DOSES OF METHYL MERCURY CHLORIDE PER KILOGRAM OF BODY WEIGHT AND RADIATION IN R

		Radiation Dose		
		High (300)	Low (150)	Control (0)
Mercury Dose	High 6 mg/kg	Group 1	----	Group 3
	Low 3 mg/kg	----	Group 6	----
	Control	Group 4	Group 2	Group 5

RESULTS

All animals administered methyl mercury, alone or as a coinsult, vomited within 2 hours of administration of the agent. Anoxeria of 24-72 hours duration was also observed. The animals were lethargic and exhibited general signs of discomfort during this period. This was followed by a prompt return to normal behavior. Activity of the animals receiving radiation alone could not be distinguished from that of the controls.

Only one animal died; one female which received the high dose of methyl mercury alone began exhibiting typical symptoms of the terminal syndrome described earlier (Hoskins and Hupp) and died 2 days later, 7 days after treatment.

The number of correct responses obtained in the WGTA was reduced in all animals that received the high dose of methyl mercury, either alone or as a coinsult, the first day after treatment. One of the two females which received the low coinsult also had a decreased number of correct responses at this time. The two females that received methyl mercury only continued to have fewer correct responses. The female that died performed normally 4 days after treatment, then failed to perform subsequent days until death while exhibiting obvious signs of methylmercury-induced illness. The female that survived returned to normal by 9 days after treatment. No significant depression in the number of correct responses was observed in the other groups after the first day. For an unexplained reason, one of the two controls stopped performing at all a few days after treatment of the treated animals.

The latency (period of time for the animal to choose a stimulus object, beginning when the tray touched the transfer cage and ending when the animal touched a stimulus object with his hand) appeared to be affected for a longer period of time than the number of correct responses. The animals receiving the coinsult had increased latency for 12 and 13 days; the animal that survived the mercury only had increased latency for 7 days while the one that died had increased latency all the days she performed. Thus, in contrast to the number of correct responses, latency seemed to be affected more in the coinsulted animals.

One of the animals receiving the low coinsult had increased latencies for 9 days after treatment, though no significant changes were observed in the other one receiving this treatment. One of the two animals receiving 300 R radiation only had a slight increase in latency on days 1, 2, 13, and 14 after treatment with the other showing no change; the one animal receiving 150 R also showed no change. One control exhibited very constant latencies, while the one that ceased to perform had increased latencies for the last several days that she performed.

The duration of response (from the end of latency to when the subject put the raisin in her mouth) was generally similar to that for the number of correct responses but the data seemed to be more variable than for the other two parameters. In agreement with the other two paramenters, the two groups that received the large dose of methylmercury showed the only consistent treatment effect. One animal receiving the coinsult generally had increased duration of response through 20 days after treatment, then returned to pre-treatment values; the other had increased duration through day 12, then generally had some increase through day 25. The animal surviving the high mercury treatment generally had increased duration for 8 days after treatment, while the decedent had increased duration all the days that she responded following treatment.

One female receiving the low coinsult had increased duration of response through day 5, then returned to pre-treatment values, while the other member of this group showed no change. One animal receiving 300 R had several days with high duration of response, but exhibited no consistent pattern; the other did not show any change, responding with the greatest consistency shown for this parameter of any of the animals tested. One control and the animal receiving 150 R only had very consistent responses; as with the latency responses, the control that quit responding had increased duration for several days before completely stopping.

DISCUSSION

Response of the mercury-treated females was similar to that of other monkeys tested previously. As previously observed, some variation existed in groups receiving the same treatment. In this case, marked differences were observed in the group receiving the high mercury dose and in the low coinsult group. In the former, one female died while the other recovered and exhibited no persistent symptoms. In the latter group, one female exhibited changes for a few days and then returned to normal while the other showed no effect.

The results obtained in this and previous studies indicate varying thresholds for the various effects produced by methylmercury, with individual differences causing different thresholds. The sensitivity for the various effects would appear to be vomiting > transient lethargy > anorexia > performance decrement in WGTA > reduced general activity > blindness > death. With regard to the latter two parameters, some animals receiving a dose that was lethal to one or more animals receiving a similar dose became blind while others did not. In the case of females, no blind animals were produced, while several males became blind.

Coinsult effects were generally similar to those observed in rats. In this study, the coinsult groups performed very similarly to those receiving the same dose of mercury only. In some parameters measured, the high coinsult groups appeared slightly more adversely affected than mercury only; for other parameters, the reverse appeared to be the case. Sample size was too small to draw extensive conclusions from with regard to lethality; no major additivity of the two insults is possible, however, since both animals receiving the high coinsult survived while only one of the two mercury-only animals survived. Based on this and previous studies, 6 mg methylmercuric chloride/kg body weight appears to be approximately an LD_{50} dose. The LD_{50} for radiation has not been accurately determined; based on previous hematology studies in this laboratory, the 300 R dose was considered barely sublethal. Thus if significant additivity of the two insults exists, one or both animals receiving the high coinsult should have died.

The behavior of the control animal which failed to continue to perform is unexplained. A total of 25 animals have been tested in our apparatus under the same conditions, and this is the only one which has failed to continue to perform. The test procedure may thus considered to be very reliable.

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